CHAPTER

1

Introduction

Pathology and the safety assessment of new medicines

Evaluation of the pathological alterations induced in laboratory animals by novel drugs represents the cornerstone of their safety assessment before they can be first tried in patients. This preliminary assessment, which is based largely on conventional histopathological techniques, represents a major contribution to the development of new treatments for both human and animal diseases.[1]

Although there have been many changes in the details of study design and conduct, the principles of drug testing prior to trial in humans are the same as those expounded by Geiling and Cannon after they studied the pathological effects and causes of death of patients treated with a toxic elixir of sulfanilamide over 60 years ago (Table 1.1).[2] The basic paradigm of dosing laboratory animals with various doses of a new drug for increasing periods of time accompanied by careful clinical observations, biochemical and hematological monitoring followed by histopathological examination of the tissues remains essentially unaltered and has withstood the test of time. The pathologist is required not only to evaluate alterations to organs and tissues and any relationship that they might have to drug treatment but also to assess likely relevance any treatment-related findings might have for patients.

The use of animals to study the pathological effects of chemicals and therapeutic agents has a long history. In the 18th century Morgagni reported his attempts to compare pathological changes produced by accidental ingestion of chemicals such as arsenic by people with those induced by administration to animals.[3] A thorough and systematic review of pathology induced by toxins in humans and animals was published by Orfila as long ago as 1815.[4] Although in the modern era drug safety evaluation has been widely practiced in rodent and non-rodent species since before the Second World War, there have been very few critical comparisons of the effects of drugs in humans and these laboratory animals. Much potentially useful information still resides in archives of pharmaceutical companies and government agencies. Nevertheless, the available data suggest that the traditional approach using

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TABLE 1.1 Principles of Drug Testing before Trials in Humans as Defined in 1938 by Geiling and Cannon[2]

1.	Exact composition of drug should be known; if not, method of preparation
2.	Acute toxicity studies in animals of different species
3.	Chronic toxicity experiments at varying doses in different species for cumulative effects
4.	Careful and frequent observations of animals, to develop a composite picture of clinical effects
5.	Careful pathological examination of tissues with appropriate stains
6.	Effects of drugs on excretory or detoxifying organs, especially kidney and liver
7.	Rate of absorption and elimination, path and manner of excretion, concentration in blood and tissues at varying times
8.	Possible influence of other drugs and foodstuffs
9.	Careful examination for any idiosyncrasies or untoward reactions

experimental pharmacology alongside conventional toxicology studies with pathology is usually sufficient to predict important adverse effects and to support the safe conduct of the first clinical studies in humans.[1] Indeed, dosing a rodent and non-rodent species with a new drug up to one month identifies over 90% of adverse effects that will ever be detected in conventional animal studies. However, these animal studies do not predict all adverse drug effects that can occur in clinical practice and there remains significant over- and underprediction of human toxicity. Overall the true positive concordance rate (sensitivity) is of the order of 70% with 30% of human toxicities not predicted by safety pharmacology or conventional toxicity studies.[5] Moreover, this concordance varies between different organs and tissues. Therefore each drug-induced pathological finding needs to be assessed on a case-by-case basis for its likely clinical relevance. Moreover, for some systems, histopathology remains crucial, for others it is of lesser importance. For example, animal studies are poor predictors of subjective neurological symptoms but histopathological examination of the nervous system in laboratory animals treated with cancer drugs detects potential serious neurotoxic effects in humans. Likewise pathological examination of the skin in conventional toxicity studies does little to identify important adverse skin hypersensitivity reactions in humans, whereas there appears to be an excellent correlation between the adverse effects in subcutaneous and intramuscular injection sites between animals and humans.[1] Animal studies seem to overpredict renal and hepatic toxicity but there is generally a good correlation for gastrointestinal effects. Histopathology still seems to represent one of the most sensitive techniques to detect effects on the reproductive system.[6] Nevertheless, the pathologist also needs to be aware that some minor inflammatory alterations in certain organs such as the liver may have greater significance for the use of a drug in humans than particular types of severe damage such as subendocardial necrosis in the myocardium mediated by exaggerated hemodynamic effects.

Treatment-induced findings in conventional toxicity studies found in different laboratory animal species also have variable prognostic value for humans. Although the data are fragmentary, findings in beagle dogs studies appear overall to be better predictors of human adverse effects than data from rodents or surprisingly from primates.[1] Dog gastrointestinal and cardiovascular physiology appears to model particularly well for humans.[7,8]

Another long-standing problem highlighted by the cyclooxygenase 2 (COX-2) inhibitors is the adverse interaction of some therapies with specific human diseases. COX-2 inhibitors were used for inflammatory disorders because of perceived lower side effect profile on the gastrointestinal tract compared with conventional drugs. This benefit was outweighed by an increased incidence of cardiovascular disease in some patients, although withdrawal of such drugs from the market may have reduced the availability of effective treatments for some arthritis patients.[9] Similar concerns about an increase in ischemic cardiovascular events with rosiglitazone compared with other non-thiazolidinedione antidiabetic agents has also been the basis for limiting the use of this effective drug by the drug regulatory authorities.[10] Such effects are difficult if not impossible to predict from the usual clinical trials let alone conventional toxicity studies. Unfortunately the detection of an increased incidence of a common event such as heart attack or stroke is difficult in patients for it requires a high index of suspicion even though it may have a big impact on public health.[11,12] Such interactions usually require randomized controlled trials specifically designed to look for such risks.[11] It has to be remembered that aspirin was in use for over 100 years before it became generally acknowledged about 30 years ago to be associated with Reye's syndrome, a devastating hepatic toxicity in children.[13] Although the precise mechanism involved in Reye's syndrome is unknown it is often preceded by a viral syndrome, usually varicella, gastroenteritis, or an upper respiratory or tract infection such as influenza and it shows a strong epidemiologic association with the ingestion of aspirin.

Veterinary medicines

Similar principles apply to the development and the safety assessment of new medicines for animals, although assessment of environmental impact and residue studies are also required for consumer safety for food-producing animal medicines. While assessment of the relevance of drug-induced pathological findings in laboratory animals requires extrapolation to a wider range of other species, the task is often aided by the ability to conduct toxicity studies at multiples of the therapeutic dose in the target species – but again supported by histopathological examination.[14]

Toxicological screening

Screening compounds to select the least toxic in a series of chemicals has a long pedigree. In 1909 Paul Ehrlich, looking for a cure for infectious disease, screened a large number of arsenic-containing compounds in mice, guinea pigs and rabbits.[15] He discovered that one compound #606 not only killed the syphilis microbe but also cured rabbits with syphilis without causing death. This chemical was marketed as the first effective remedy for syphilis under the name of *Salvarsan*. Gerhard Zbinden and colleagues made a convincing case for flexible, targeted toxicity studies of a series of related chemicals using standard reference agents and small numbers of animals for short periods of time in the selection of the least toxic candidate new drugs.[16] These studies are quite widely practiced but they require careful design, critical selection of models and careful evaluation of pathology. In this respect, pathological evaluation of important organs such as liver and

kidney in pharmacology studies conducted in disease models can also provide insights to potential toxicity issues.

Carcinogenesis assessment

The evaluation of the carcinogenic potential of drugs designed for long-term use is often seen as where the pathologist 'comes into his or her own'. Carcinogenicity studies require the careful diagnosis of diverse tumors and preneoplastic lesions that can occur in rodents. However, the contribution of these studies to human safety is not clear cut. About half of the drugs that have been developed over the past two decades have shown tumorigenicity in rodents.[17] If a few genotoxic drugs are excluded, the majority appear to have induced tumors as a consequence of exaggerated or unwanted pharmacodynamic effects at high doses which have not precluded their use in patients for treatment of disease.

Various modes of action have been linked to these tumor types although the underlying mechanisms are often unclear.[18–22] However, from a pathological perspective, non-relevant tumors tend to occur at high doses where there is histological evidence of persistent cellular toxicity, exaggerated pharmacodynamic effects or other perturbations of homeostasis.[23] By contrast, the evidence of tumorigenic response from dosing a range of potent DNA reactive (genotoxic) carcinogens to rodents suggests that there is clear histological evidence of an increase in malignancy in induced tumors compared with tumors that develop spontaneously. Evidence of a malignant phenotype is the presence of metastases distant from the primary tumor site rather than cytological appearances alone. Moreover there is usually a much earlier age of onset compared with tumors that develop spontaneously and those that follow administration of non-DNA reactive chemicals. A review of the *National Toxicology Program* (NTP) database also suggested that potent genotoxic carcinogens produce tumors in characteristic multiple sites in rodent studies.[24] Much relevant information is scattered in the pathology literature, although there have been a number of pathology reviews of rodent tumor types of questionable significance to humans.[18,25–27]

In view of these difficulties as well as the resources needed and time involved in conducting a traditional two-year carcinogenicity study in both rats and mice, other approaches have been proposed. It has long been argued that the traditional mouse carcinogenicity study adds little or nothing to the evaluation of carcinogenicity and is consequently a redundant test.[28] Monro suggested that, because of improved understanding of rodent tumorigenesis, a single study of 12 to 18 months' duration in rats alone would be sufficient to identify potential human carcinogens.[29] More recent comparisons of results from chronic toxicity studies with carcinogenicity studies performed on a large number of pharmaceutical agents has also suggested that six-month and 12-month toxicity studies are reasonable predictors of tumorigenic outcome in two-year studies.[30,31] Cohen has even suggested that critical evaluation of cellular findings in animal studies of merely 13 weeks' duration can identify many of the chemicals that go on to produce tumors in long-term studies.[32] In fact, the prudent pathologist has always evaluated pathology findings in chronic toxicity studies in this way to try to predict tumorigenicity and avoid the unexpected at the end of two-year bioassays. This has become essential as part of the

evaluation of the potential carcinogenicity of biotechnology derived drugs where traditional two-year studies may not be feasible.[33]

In view of the fact that the traditional carcinogenicity study in the mouse contributes little to the evaluation of carcinogenic potential, short-term studies in genetically engineered mice have been used as substitutes. Most commonly used alternatives models have been the mouse heterozygous for $p53^{+/-}$ tumor suppressor gene and the rasH2 mouse model which carries the human c-Ha-*ras* oncogene in addition to the endogenous murine Ha-*ras* oncogene.[34] However, uncertainties remain and results have been somewhat mixed so conventional carcinogenicity studies of rats and mice are still widely performed.

Nevertheless, whatever the precise protocol, species or strain of rodent used, the pathologist remains essential in the *in vivo* assessment of tumorigenicity. It remains largely the role of the pathologist to evaluate the findings *in vivo* studies of carcinogenesis provide explanation for any tumor development and indicate likely relevance or lack of relevance for humans. Various frameworks have been devised to aid in the assessment of tumor relevance.[19,20]

Comparative pathology

Another issue for the pathologist is that of comparative pathology. Over recent years there has been renewed interest in the synergy between animal and human diseases emerging from the study of receptors, mediators and genes common to both.[35,36] However, few pathologists have attempted critical and systematic reviews of animal and human diseases. Still pertinent today is a comment made by the British pathologist Willis who studied both animal and human tumors nearly 50 years ago that 'more use should be made of the pathological material passing through the hands of veterinarians, breeders and slaughtermen, most of which is wasted'.[37]

Lack of critical correlation means that terminology common to both laboratory animal and human pathology can mislead. A term used for a rodent lesion may reflect pathology of a quite different biological behavior in humans. For example, rat mammary carcinomas have a different biological behavior to the common breast carcinomas in women. Mouse pulmonary tumors are slow growing expansive lesions whereas common pulmonary cancers are highly invasive with poor prognosis in humans. Some conditions are particularly common in rodents but rare in humans, for example histiocytic sarcoma which has a common but variable incidence in rats and mice. Moreover, the pathological response in animals to the same adverse effect may be different to that occurring in humans. Basal cell carcinomas of the skin are the most common cancers associated with exposure to ultraviolet light in humans but squamous carcinoma is the principal tumor type induced in animals.[38]

It is also worth remarking on the different approach to the diagnosis of neoplastic lesions in experimental animals and humans. In the diagnosis of human neoplasms, knowledge of clinical progression, ability to image and biopsy sequentially means that many proliferative lesions which may be nodular and displace surrounding tissues or show cytological atypia may be considered non-neoplastic in nature. This background information is usually lacking in experimental situations where diagnoses are almost

always based on histological and cytological characteristics alone. Hence for this reason diagnoses made for laboratory animals may not always equate to lesions of the same name in humans.

Techniques in pathology

Over the last few years a number of excellent reviews of best practice for application to the histopathology evaluation of toxicology studies have been produced. They cover basic procedures such as selection of organs for weighing, recommendations for tissue lists, blocking and sectioning procedures, data collection and peer review.[39–47]

There are also well-defined procedures for *pathology working groups* when there is dissent about diagnoses or interpretation.[45]

In addition, there are now many good reviews of special techniques applicable to toxicity testing such as recommended antibodies for use in immunocytochemistry in laboratory rodents and recombinant DNA technologies.[48–52] However, it is important that these techniques are used in a judicious manner with clear aims following careful analysis of conventional hematoxylin and eosin-stained sections.

Above all, there is no substitute for good, conventional histopathological analysis. Here also a number of recommendations for best practice are available in the pathology literature.[53] Moreover, there is now a range of publications of internationally recognized standardized nomenclatures for lesions found in most organs systems in conventional rodent studies. These are being systematically revised.[54–56]

Unfortunately there remain widespread misconceptions about the nature of the pathological evaluation. Histopathological examination is not an exercise in matching pictures. Moreover it is not simply comparing histology of organs in treated animals with those in controls for this fails to discriminate between the important and the irrelevant. It represents a careful step-by-step evaluation of tissue and cellular patterns in individual animals. This includes assessment of the size, shape, staining characteristics and organization of diverse cell and tissue components and integration of the findings into meaningful biological conclusions. By definition, good histopathology assessment includes a semi-quantitative assessment and integration of features such as cell numbers, mitoses, size of blood vessels and other structures for which the human brain still outperforms the computer. It requires an evaluation whether any observed differences between groups are likely to be a consequence of the usual variability among test animals or a consequence of fixation or processing artefact before concluding findings may be related to treatment. Some changes may simply be result of an interaction between spontaneous animal pathology and environmental factors or other experimental variables that produce apparent differences between groups without being a direct result of treatment with the test agent.

Reporting of pathology findings

Report writing represents the final but one of the most important tasks of the pathologist for which there are recommended best practices for conventional toxicity studies.[57] A report requires particular clarity as it serves a very diverse readership. On the one hand,

there are practising physicians who depend on the veracity of pathology report to design, conduct and monitor the safety of patients or volunteers in clinical trials. Some physicians have a particularly good knowledge of histopathology in their own speciality. At the other extreme are lay people, for example on ethical review committees, who will have rudimentary knowledge of pathology. Although most of the readership will lie in between these two extremes, it is salutary to remember that toxicologists and physicians in government regulatory authorities usually read the text relating to pathology findings with extreme care, whether integrated into the final document or in a stand-alone report. In addition the tabulated summaries of pathology are often reviewed with equal attention. Unclear language, inappropriate, misleading or unexplained terminology, conclusions not justified by the data, any discrepancy between text and tables may all raise unnecessary questions. Thus, clarity of the report and explanation of all findings are essential. The comments of the British writer of *1984* George Orwell remind us: 'never use a long word when a short one will do; if it is possible to cut a word out, always cut it out; never use jargon if you can think of an everyday English equivalent'.

The following chapters

The subsequent text is arranged as in previous editions into chapters on organ systems. While the main aim is to describe drug-induced pathology in laboratory animals, it also attempts where possible to comment on the likely relevance of animal findings for human patients. For this reason the text also embraces aspects of comparative anatomy and pathology and drug-induced reactions in patients. Of course it cannot be fully comprehensive. Today the information is so vast and fragmented that it is difficult to match the astonishing range of information contained in the book written by Orfila towards the end of the Napoleonic era in France.[4] He not only reviewed the data on the symptoms and autopsy alterations produced in people by a vast range of chemical and biological agents including those with therapeutic activity such as metal salts, opium, curare, ergot and snake venoms but he studied their clinical and pathological effects in animals, mostly dogs. He gave consideration to dose, route, salt form and formulation. From him we learn that the inhabitants of Edinburgh and London in the early 18th century swallowed every morning a dose of native metallic mercury mixed in oil without ill-effect to protect against gout and calculi. He confirmed the innocuity of this formulation in dogs but showed that this form of mercury could be toxic and cause death if administered in a way that allowed it to be degraded and therefore absorbed.

Ultimately, safe conduct of clinical trials depends on a sound interpretation of preclinical finding particularly pathology, based on informed judgement and realistic understanding of the limits of imperfect animal studies tempered by common sense. It is hoped that the broad overview provided in the following chapters will be helpful to readers engaged in this endeavor.

References

1. Greaves P, Williams A, Eve M. First dose of potential new medicines to humans: how animals help. *Nat Rev Drug Discov* 2004;**3**:226–36.

2. Geiling EMK, Cannon PR. Pathologic effects of elixir of sulphanilamide (diethylene glycol) poisoning. *JAMA* 1938;**111**:919–26.
3. Morgani JB. *The seats and causes of disease investigated by anatomy in five books, containing a great variety of dissections with remarks*. London: Millar, A. & Cadell, T. 1769.
4. Orfila MJB. *Traité des poisons. Tirés des regnes minéral, végétal et animal, ou Toxicologie générale, considerée sous les rapports de la physiologie, de la pathologie et de la médicine légale*. Paris: Crochard; 1815.
5. Olsen H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and animals. *Regul Toxicol Pharmacol* 2000;**32**:56–67.
6. Takayama S, Akaike M, Kawashima K, Takahashi M, Kurokawa Y. Study in Japan on optimal treatment period and parameters for detection of male fertility disorders in rats induced by medical drugs. *J Am Coll Toxicol* 1995;**14**:266–92.
7. Dressman JB. Comparison of canine and human gastrointestinal physiology. *Pharmacol Res* 1986;**3**:123–31.
8. Mitchell AR. Hypertension in dogs: the value of comparative medicine. *J R Soc Med* 2000;**93**:451–2.
9. Olsen NJ. Tailoring arthritis therapy in the wake of the NSAID crisis. *N Engl J Med* 2005;**352**:2578–80.
10. Woodcock J, Sharfstein JM, Hamburg M. Regulatory action on rosiglitazone by the U.S. Food and Drug Administration. *N Engl J Med* 2010;**363**:1489–91.
11. Drazen JM. COX-2 inhibitors – a lesson in unexpected problems. *N Engl J Med* 2005;**352**:1131–2.
12. Trontell A. Expecting the unexpected – drug safety, pharmacovigilance and the prepared mind. *N Engl J Med* 2004;**351**:1385–7.
13. Monto AS. The disappearance of Reye's syndrome – a public health triumph. *N Engl J Med* 1999;**340**:1423–4.
14. Woodward KN. Veterinary pharmacovigilance. Part 6. Predictability of adverse reactions in animals from laboratory toxicology studies. *J Vet Pharmacol Ther* 2005;**28**:213–31.
15. Drews J. Paul Ehrlich: Magister Mundi. *Nat Rev Drug Discov* 2004;**3**:797–801.
16. Zbinden G, Elsner J, Boelsterli UA. Toxicological screening. *Regul Toxicol Pharmacol* 1984;**4**:275–86.
17. Davies TS, Monro A. Marketed human pharmaceuticals reported to be tumorigenic in rodents. *J Am Coll Toxicol* 1995;**14**:90–107.
18. Cohen SM. Human carcinogenicity risk evaluation: an alternative approach to the two-year bioassay. *Toxicol Sci* 2004;**80**:225–9.
19. Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD, et al. A framework for human relevance analysis of information on carcinogenic modes of action. *Crit Rev Toxicol* 2003;**33**:591–653.
20. Boobis AR, Doe JE, Heinrich-Hirsch B, Meek ME, Munn S, Ruchirawat M, et al. IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit Rev Toxicol* 2008;**38**:87–96.
21. Hoenerhoff MJ, Hong HH, Ton T-v, Lahousse SA, Sills RC. A review of the molecular mechanisms of chemically induced neoplasia in rat and mouse models in National Toxicology Program bioassays and their relevance to human cancer. *Toxicol Pathol* 2009;**37**:835–48.
22. Holsapple MP, Pitot HC, Cohen SH, Boobis AR, Klaunig JE, Pastoor T, et al. Mode of action in relevance of rodent liver tumors to human cancer risk. *Toxicol Sci* 2006;**89**:51–6.
23. Grasso P. Experimental liver tumors in animals. *Baillieres Clin Gastroenterol* 1987;**1**:183–205.
24. Gold LS, Manley NB, Slone TH, Ward JM. Compendium of chemical carcinogens by target organ: results of bioassays in rats, mice, hamsters, dogs and monkeys. *Toxicol Pathol* 2001;**29**:639–52.
25. Alison RH, Capen CC, Prentice DE. Neoplastic lesions of questionable significance to humans. *Toxicol Pathol* 1994;**22**:179–86.
26. Grasso P, Sharratt M, Cohen AJ. Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. *Annu Rev Pharmacol Toxicol* 1991;**31**:253–87.
27. Williams GM, Iatropoulos MJ, Enzmann HG. Principles of testing for carcinogenic activity. In: Wallace Hayes A, editor. *Principles and methods of toxicology*. Philadelphia, PA: Taylor and Francis; 2008. p. 1265–316.
28. Schach von Wittenau M, Estes PC. The redundancy of mouse carcinogenicity bioassays. *Fundam Appl Toxicol* 1983;**3**:631–9.
29. Monro A. How useful are chronic (life-span) toxicology studies in rodents in identifying pharmaceuticals that pose a carcinogenic risk to humans? *Adverse Drug React Toxicol Rev* 1993;**12**:5–34.

30. Reddy MV, Sistare FD, Christensen JS, DeLuca JG, Wollenberg GK, DeGeorge JJ. An evaluation of chronic 6- and 12-month rat toxicology studies as predictors of 2-year tumor outcome. *Vet Pathol* 2010;**47**:614–29.
31. Jacobson-Kram D. Cancer risk assessment approaches at the FDA/CDER: is the era of the 2-year bioassay drawing to a close? *Toxicol Pathol* 2010;**38**:169–70.
32. Cohen SM. An enhanced 13-week bioassay: an alternative to the 2-year bioassay to screen for human carcinogenesis. *Exp Toxicol Pathol* 2010;**62**:497–502.
33. Vahle JL, Finch GL, Heidel SM, Hovland DN, Ivens I, Parker S, et al. Carcinogenicity assessments of biotechnology-derived pharmaceuticals: a review of approved molecules and best practice recommendations. *Toxicol Pathol* 2010;**38**:522–53.
34. Long GG, Morton D, Peters T, Short B, Skydsgaard M. Alternative mouse models for carcinogenicity assessment: industry use and issues with pathology interpretation. *Toxicol Pathol* 2010;**38**:43–50.
35. Lemon R, Dunnett SB. Surveying the literature from animal experiments. Critical reviews may be helpful – not systematic ones. *Br Med J* 2005;**330**:977–8.
36. Mitchell AR. What could Dr Finlay and Mr Herriot learn from each other? Comparison of human and animal diseases can benefit patients of all species. *Br Med J* 2005;**331**:1220–1.
37. Willis RA. *Pathology of tumours*. London: Butterworths; 1960.
38. de Gruijl F, Forbes PD. UV-induced skin cancer in a hairless mouse model. *Bioessays* 1995;**17**:651–60.
39. Bregman CL, Adler RR, Morton DG, Regan KS, Yano BL. Recommended tissue list for histopathologic examination in repeat-dose toxicity and carcinogenicity studies: a proposal of the society of toxicologic pathology (STP). *Toxicol Pathol* 2003;**31**:252–3.
40. Ruehl-Fehlert C, Kittel B, Morawietz G, Deslex P, Keenan C, Mahrt CR, et al. Revised guides for organ sampling and trimming in rats and mice – Part 1. *Exp Toxicol Pathol* 2003;**55**:91–106.
41. Kittel B, Ruehl-Fehlert C, Morawietz G, Klapwijk J, Elwell MR, Lenz B, et al. Revised guides for organ sampling and trimming in rats and mice – Part 2. *Exp Toxicol Pathol* 2004;**55**:413–31.
42. Morawietz G, Ruehl-Fehlert C, Kittel B, Bube A, Keane K, Halm S, et al. Revised guides for organ sampling and trimming in rats and mice – Part 3. *Exp Toxicol Pathol* 2004;**55**:433–49.
43. Mikaelian I, Nanney LB, Parman KS, Kusewitt DF, Ward JM, Naf D, et al. Antibodies that label paraffin-embedded mouse tissues: a collaborative endeavor. *Toxicol Pathol* 2004;**32**:181–91.
44. Sellers RS, Mortan D, Michael B, Roome N, Johnson JK, Yano BL, et al. Society of Toxicologic Pathology position paper: Organ weight recommendations for toxicology studies. *Toxicol Pathol* 2007;**35**:751–5.
45. Ward JM, Hardisty JF, Hailey JR, Streett CS. Peer review in toxicologic pathology. *Toxicol Pathol* 1995;**23**:226–34.
46. Greaves P. Pathological techniques in toxicology. In: Ballantyne B, Marrs TC, Syversen T, editors. *General and applied toxicology*, Vol. 2. Chichester, United Kingdom: Wiley; 2009.
47. Morton D, Sellers RS, Barale-Thomas E, Bolon B, George C, Hardisty JF, et al. Recommendations for pathology peer review. *Toxicol Pathol* 2010;**38**:1118–27.
48. Gant TW, Greaves P, Smith AG, Gescher AJ. Toxicogenomics applied to understanding cholestasis and steatosis in the liver. In: Borlak J, editor. *Handbook of toxicogenomics*. Weinheim: Wiley-VCH; 2005. p. 369–94.
49. Kunder S, Calzada-Wack J, Hölzlwimmer G, Müller J, Kloss C, Howat W, et al. A comprehensive antibody panel for immunohistochemical analysis of formalin-fixed, paraffin-embedded hematopoietic neoplasms of mice: analysis of mouse specific and human antibodies cross-reactive with murine tissue. *Toxicol Pathol* 2007;**35**:366–75.
50. Randall KJ, Pearse G. A dual-label technique for the immunohistochemical demonstration of T-lymphocyte subsets in formalin-fixed, paraffin-embedded rat lymphoid tissue. *Toxicol Pathol* 2008;**36**:795–804.
51. Foster WR, Chen S-J, He A, Truong A, Bhaskaran V, Nelson DM, et al. A retrospective analysis of toxicogenomics in the safety assessment of drug candidates. *Toxicol Pathol* 2007;**35**:621–35.
52. Malarkey DE, Maronpot RR. Polymerase chain reaction and in situ hybridization: applications in toxicological pathology. *Toxicol Pathol* 1996;**24**:13–23.
53. Crissman JW, Goodman DG, Hildebrandt PK, Maronpot RR, Prater DA, Riley JH, et al. Best practices guideline: toxicologic histopathology. *Toxicol Pathol* 2004;**32**:126–31.

54. Vahle J, Bradley A, Harada T, Herbert R, Kaufmann W, Kellner R, et al. The International Nomenclature Project: an update. *Toxicol Pathol* 2009;**37**:694–7.
55. Renne R, Brix A, Harkema J, Herbert R, Kittel B, Lewis D, et al. Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. *Toxicol Pathol* 2009;**37**:5S–73S.
56. Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, et al. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol* 2010;**38**:5S–81S.
57. Morton D, Kemp RK, Francke-Carroll S, Jensen K, McCartney J, Monticello TM, et al. Best practices for reporting pathology interpretations within GLP toxicology studies. *Toxicol Pathol* 2006;**34**:806–9.

CHAPTER

2

Integumentary System

SKIN AND SUBCUTANEOUS TISSUE

Skin lesions are among some of the most common adverse reactions to drugs in clinical practice. Although it is difficult to ascertain their true incidence because of lack of data in the outpatient population, morbilliform rashes, urticaria and generalized pruritus have been reported to occur in up to 2 or 3% of hospitalized patients.[1–4] They may be one of the largest proportion of drug-related causes of emergency department visits and hospital admissions.[5] Skin reactions tend to be more frequent in elderly patients treated with multiple drugs and in females. However, the skin is also one of the more common targets for drug reactions in children.[6] Non-steroidal anti-inflammatory drugs, the penicillins and trimethoprim-sulphamethoxasole are associated with a particularly high rate of adverse skin reactions although newer drugs have produced some novel reaction patterns.[7] A large proportion of these skin reactions appear to be a result of hypersensitivity or other immune mediated reactions.[8,9] While most of these reactions are not severe, some skin reactions notably toxic epidermal necrolysis or Lyell's syndrome can be life threatening if treatment is not discontinued.[10]

Histologically, drug reactions are associated with a variety of inflammatory disease patterns in the skin and subcutaneous tissues. No pattern appears to be specific for a particular drug although presence of eosinophils can be a characteristic feature.[11] The most common form which has been linked to systemically administered drugs is characterized by a perivascular and mainly lymphocytic inflammatory infiltrate within the dermis or subcutaneous tissues.[4] As the skin only develops relatively non-specific adverse reaction patterns to a diverse range of adverse stimuli it has not often been possible to delineate the precise pathogenic mechanisms involved.

The skin also represents an important target for anticancer drugs by virtue of its high metabolic rate. Diverse types of skin reactions are reported although it is difficult to attribute to particular drugs because of multiple therapies and a range of other skin lesions of other types found in cancer patients.[12]

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Cutaneous drug reactions are particularly common in patients with human immunodeficiency virus (HIV) infection and their incidence increases as immune function deteriorates.[13] The incidence of skin carcinomas increases with the duration of immunosuppressive therapy particularly in white transplant recipients.[14]

The prevalence of drug-induced skin reactions is perhaps not surprising when the skin is the largest organ of the body which not only acts as a physical barrier but also has innate and adaptive immune defense functions. Squamous epithelial cells act not only as a physical barrier but also as an early warning system which releases cytokines and chemokines in response to injury. Immune cells present in the epidermis include specialized dendritic cells known as Langerhans cells and intraepithelial lymphocytes. Immune system cells present in the normal dermis include dendritic cells, mast cells and a small number of cutaneous lymphocyte memory T cells. Dermal post-capillary venules express adhesion molecules that support the margination and emigration of memory T cells into non-inflamed skin and direct recruitment of neutrophils, eosinophils and natural killer cells in inflammatory states.[15] The adaptive immune system involving B and T lymphocytes incorporates systems that bring antigens encountered in the skin to antigen presenting cells and naive T cells in the local lymph nodes. It is these factors that underlie the fact that vaccination through the skin is most effective at stimulating skin homing effector cells whereas vaccination at other sites more efficiently generate effector memory T cells that home to other sites.[15]

Despite advances in knowledge of the role of skin in the modulation of cutaneous immune responses, the evidence suggests that conventional animal toxicity studies predict the most common adverse reactions in the skin only poorly.[16] This is consistent with the idiosyncratic or unpredictable nature of many drug-induced skin reactions in people. Adverse skin effects may only become evident in large-scale clinical trials or in general clinical practice following marketing of new drugs. In the study by Olson and colleagues comparing preclinical and clinical data of new drugs in development it was shown that, although relatively few agents of those reviewed (less than 10%) developed skin adverse reactions in clinical trials, animal studies had shown skin changes in only about a third of these cases.[17]

However, preclinical studies are helpful in the indication of certain types of potential skin effects in human patients. The local irritancy of topically applied substances in animal models appears to be reasonably predictive of irritancy potential in humans. Antiproliferative drugs such as bleomycin that target the epidermis directly produce skin changes in both humans and animals. Exaggerated pharmacodynamic changes such as seen with epidermal growth factor receptor inhibitors have been observed in animal studies as well as in patients.[18–20] Topical application of drugs designed to activate components of the immune system may also produce pharmacodynamics changes in both humans and animals.[21] Compounds with a high affinity for melanin have been associated with skin changes in humans and therefore new drugs that bind to melanin or inhibit enzymes associated with melanin biosynthesis should be assessed carefully in animal models for toxicity in melanin-containing organs. Cutaneous blood vessels or sebaceous glands can also be the targets of drug treatment. Adverse effects may be seen on wound healing and this can be assessed in animals.[22] The dorsum of the rat appears to be one of the most commonly used sites for the study of wound healing.[23] The skin also exhibits

alterations that are manifestations of systemic pathological processes. For instance, failure of blood coagulation can lead to purpura and bleeding. Pituitary and thyroid disorders, changes in endocrine pancreas and derangement of calcium balance are also associated with cutaneous manifestations.[24]

The inflammatory reaction to substances injected or implanted into the subcutaneous tissues of animals correlates reasonably well with the inflammatory response in humans, although not necessarily clinical tolerance. The subcutaneous tissue is also employed as a route for immunization as it has the advantage of acting as a depot site for cytokines so allowing a longer persistence of antigen at the site of deposition. However, it has been shown in mice that microparticulate vaccine delivery systems require lower doses and result in a greater immune response when injected by the intradermal route compared to the subcutaneous injection.[25] It was argued that this is a result of an increased probability of interaction with the immune cells present in the skin compared with subcutaneous tissue.

Hair follicle

The hair follicle can be a target for therapeutic agents.[4,18] Although they vary in size and shape depending on location, the basic structure of the hair follicle is similar – rapidly proliferating matrix cells in the hair bulb and a hair shaft composed of intermediate filaments and associated proteins enveloped by a dermal sheath. The dermal papilla, located at the base of the follicle and comprised of specialized fibroblasts, is believed to be important in the control of matrix cells and consequently size of the hair. The cells of the outer root sheath normally display a number of keratins, adhesion molecules, cytokines and growth factor receptors that are different from those expressed by epidermal cells.[26] Hair follicle stem cells reside in the upper part of the dermal sheath in the so-called *bulge* which is prominent in mice.[27,28] These factors may partly explain why the hair follicle can be more sensitive to some therapeutic agents than the epidermis itself.

Each hair follicle cycles continuously through three stages: growth (*anagen*), involution (*catagen*) and rest (*telogen*). Many growth factors are important for normal hair follicle development and cycling.[26] The importance of the epidermal growth factor (EGR) receptor system has been recently recognized following studies with knockout mice lacking transforming growth factor α, the major ligand for the EGF receptor. These mice have abnormal hair follicle development.[29]

Species differences

The principle barrier function of the skin resides in the stratum corneum and there are considerable species and regional differences in the thickness of this layer (Figure 2.1). In rats the dorsal skin, which is often used as a site to administer compounds topically or by injection, has been shown to have regional variations in thickness and a greater thickness in males compared with females.[30] Humans, like pigs, possess a thicker stratum corneum than the rabbit, guinea pig or mouse. The practice of shaving the skin of test species may influence absorption because this can affect the natural protective capacity of animal skin that is partly provided by dense hair cover. In general, skin penetration of test substances is a reflection of the properties of the inert stratum corneum and differences in the physicochemical characteristics of the test substances such as lipid/water partition coefficient.

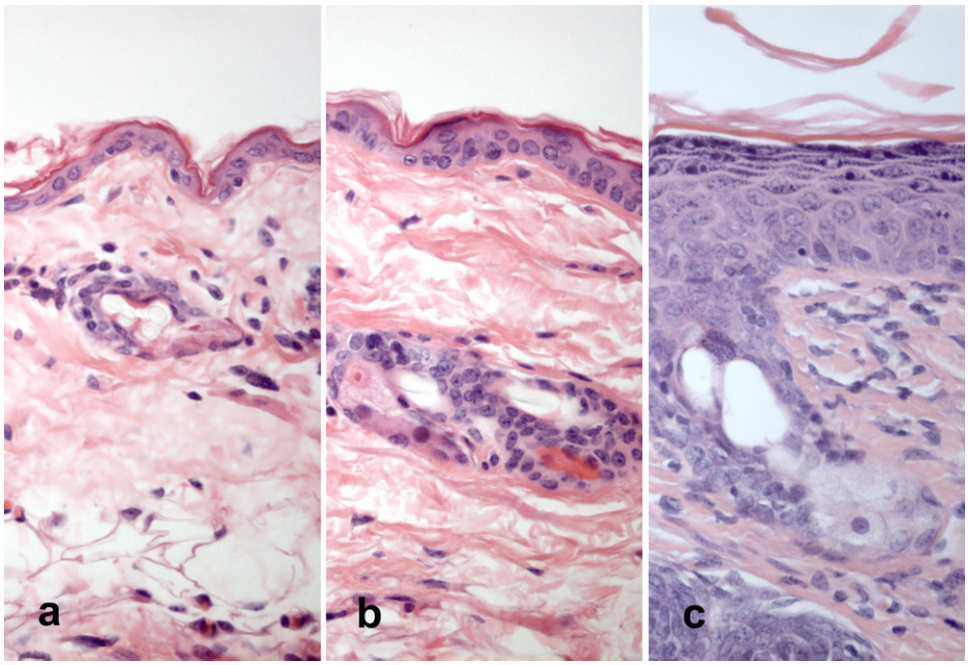

FIGURE 2.1 *Panel a:* Normal skin from the abdomen of an FVB/N mouse. *Panel b:* Normal skin from the back of an FVB/N mouse. The epidermis is thicker on the back than the abdomen. *Panel c:* Epidermal hyperplasia on the back of an FVB/N mouse as a result of the daily local application of TPA and acetone for two weeks. There is reactive hyperplasia of the epidermis. Epidermal cells are enlarged and there is a prominent keratohyalin layer. All at the same magnification (H&E ×280).

The pH values of skin vary considerably between different mammalian species. Although the functional consequences of skin pH have not been fully explored, it appears to influence barrier function and microbial growth. Human skin is generally more acidic than most laboratory animals and the dog possesses one of the highest pH of all mammalian species.[31]

On the basis of *in vivo* studies with various labeled chemicals, it has been shown that permeability of animal skin can be ranked in decreasing order of permeability: rabbit, rat, pig and human with the skin of the miniature pig skin possessing the closest permeability characteristics to that of human skin.[32] A review of percutaneous absorption of 2,4-dichlorophenoxyacetic acid showed that mouse, rat and rabbit absorption measurements all tended to be higher than humans whereas rhesus monkeys generally showed absorption profiles in the same range as humans.[33] Another comparative study of the percutaneous absorption of C_{14} radiolabeled benzoic acid, benzoic acid sodium salt, caffeine and acetylsalicyclic acid on the backs of hairless Sprague-Dawley rats and several anatomic sites in people has shown a similar rank order in the absorption of the molecules. Although the ratios of absorption between rats and the different sites in humans were different, they remained constant.[34] These results suggested that by careful control of the

conditions of application such as area, dose, vehicle and contact time, it should be possible to predict the absorption of a compound in humans. However, it must be remembered that only normal intact skin remains relatively impermeable and loss of integrity of the epidermal barrier as a result of trauma or disease processes profoundly affect the absorption of foreign substances.

There may also be significant biotransformation of topically applied substances by the viable epidermis and this activity shows considerable species variation.[35] Furthermore, increased exposure of the underlying connective tissue, skeletal muscle and joints to high concentrations of therapeutic substances administered topically may also occur.[36] This factor has been exploited for therapy of soft tissues, but may need to be considered in dermal toxicity studies. In this context a morphological difference which may influence absorption is the much more profuse dermal vasculature in humans compared with laboratory animals.[37]

The histological pattern and cell types involved in cutaneous delayed hypersensitivity reactions appears to vary among different species.[38] For example, rats and mice produce principally a monocytic–lymphocytic reaction whereas at the height of the response guinea pigs appear to develop a neutrophilic infiltration. There are species and regional differences in the density of antigen presenting Langerhans cells in the skin. For instance, in the mouse, Langerhans cells are far less numerous in the epidermis of the tail than on the abdomen and such differences may relate to immunological properties of different sites.[39] Epidermal Langerhans cell density has also been shown to decrease with advancing age in female BALB/C mice.[40]

Morphological differences between species, such as the presence or absence of the subcutaneous muscle layer (*panniculus carnosus*) may also influence the delivery of therapeutics administered by the subcutaneous route. This may be particularly important with biotherapeutic drugs where peptides and small proteins primarily diffuse through the blood vessel walls directly into capillaries, whereas large molecules are taken up into the more porous lymphatics.[41]

Non-neoplastic changes

Spontaneous inflammation and necrosis

Inflammation of the skin and subcutaneous tissues occurs following loss of integrity of the epidermal barrier as a result of the abrasions and minor everyday traumas occurring naturally among laboratory animals. The nature and distribution of these lesions usually allows the toxicologist to make a clear distinction between intercurrent and drug-induced changes. However, compounds which affect the proliferative or regenerative capacity of the germinal epithelium or the inflammatory response are capable of accentuating the appearance of ulcers and erosions at trauma sites. Excessive blood sampling or intravenous injection into the tails of rodents may also induce inflammation and marked scarring.[42]

Spontaneous, localized infections or infestations of the skin and soft tissues also give rise to inflammatory changes. Some systemic bacterial and viral diseases cause inflammation and necrosis of the skin and subcutaneous tissues in toxicity studies. For instance,

mouse pox or infectious ectromelia is a well-known skin infection of mice that can develop in laboratory animal colonies. It is characterized by a variable infiltration of the dermis by lymphocytes and macrophages and thickening of the overlying epidermis as a result of cell swelling or hyperplasia. Keratinocytes in the superficial epidermis and in hair follicles contain large eosinophilic cytoplasmic inclusions (Marshall bodies or type A inclusions) surrounded by clear halos, features similar to those seen in the skin of humans or other animals infected with pox viruses.[43]

Viral skin infections have been reported in primates in toxicity studies. This is well illustrated by the development of subcutaneous nodules reported in rhesus monkeys in a toxicity study as a result of spontaneous development of Yaba disease due to a poxvirus that is characterized by nodular proliferation of histiocytic cells.[44] In this condition, subcutaneous nodules are composed of polymorphic cells with granular cytoplasm and single or occasionally multiple eosinophilic or basophilic cytoplasmic inclusions of variable shape containing virus particles. Gough and colleagues[45] reported an outbreak of poxvirus infection in laboratory marmosets (*Callithrix jacchus*). In this outbreak, papular skin lesions developed over the entire body of affected animals. Lesions were characterized by acanthosis of the epithelial cells associated with full-thickness epidermal necrosis and ulceration. Eosinophilic, granular intracytoplasmic inclusion bodies showing ultrastructural evidence of brick-shaped virus particles, typical of poxviruses were described.

A potential problem with such opportunistic skin infections is that they may be potentiated by treatment with pharmaceutical agents. For example, bacterial infections originally developing in the skin in preclinical studies have been reported to progress to severe bacteremia in primates treated with drugs that suppress the immune system.[46]

Spontaneous inflammatory or thrombotic conditions of blood vessels can also involve surrounding soft tissues either as a result of ischemia or direct spread of the inflammatory process in the blood vessel wall to the adjacent tissues (see Cardiovascular System, Chapter 7).

Skin inflammation induced by topical administration

SKIN IRRITANCY

For topically administered therapy, potentially adverse skin effects are assessed by local application to the skin of laboratory animals before their use in humans. However, the exact predictive potential of animal models in the assessment of irritancy potential of therapeutic agents remains uncertain. Despite considerable efforts to identify new *in vitro* methods, none appear to be completely validated.[47] Draize-type testing using the rabbit and incorporating techniques such as hair shaving, abrading and use of occlusive patches remains widely used.[48] The albino guinea pig is also used and is believed by some authorities to react to skin irritants in a way more similar to humans than the rabbit. A model using the mouse ear has also been proposed as being particularly useful for mechanistic studies and better for more accurate measurement of tissue swelling.[49]

However, interspecies comparisons of the skin irritancy potential of chemicals have shown that neither the rabbit nor the guinea pig skin models are entirely reliable as predictive models for humans and that there may be a degree of over- or underprediction, depending on the type or potency of the irritant substances.[50–53] In general terms, most

animal models appear capable of predicting compounds which cause severe irritation in humans but uncertainties remain in the prediction of mild or moderate irritancy potential.[54]

Mechanistic studies of skin irritation induced in mice by chemical agents of different types have shown that the time course in development of inflammation is not solely due to differences in rates of penetration but also a result of differences in the nature of the induced inflammatory process.[49] Chemicals produce skin irritation through different pathways and histopathological examination may serve to show differences in the various components of the inflammatory process. Carefully timed histopathological examination can contribute to distinguishing between different vascular and cellular responses in the early phases of chemically induced skin irritation.

Histological examination of the skin affected by irritant substances shows a variable constellation of changes. Drugs or formulations that cause frank erosion or ulceration of the epidermis accompanied by acute inflammation or granulation tissue are usually not used in humans. However, inflammation may also be seen focally in controls where skin abrasion techniques have been employed. In most mild or moderate reactions, the epidermis remains intact but reactive changes occur. These include hyperkeratosis with increased prominence of the granular cell layer and acanthosis (Figure 2.1). Increased numbers of mitoses may be evident in the basal cell layer. An inflammatory infiltrate, principally lymphoid in type is usually present in the dermis. Edema fluid, increased numbers of polymorphonuclear cells, fibroblasts and increased prominence of the dermal vasculature are also seen. In view of experimental variables and tissue sampling factors, a simple semi-quantitative analysis of each of these components of the skin reaction is usually sufficient for histological assessment of primary skin irritancy. A simple scoring scheme for each feature separately is a useful semi-quantitative adjunct to visual assessment.[55]

It has also been argued that skin inflammation can be pharmacodynamically mediated by immune modulatory agents. Imiquimod, a powerful immune stimulator acting via the toll-like receptors 7 and 8 (TLR7 and TLR8), was shown to produce an inflammatory response in the skin of mice after topical application. After application for several days erythema, scaling and skin thickening developed, characterized histologically by the presence of acanthosis, parakeratosis and loss of the granular layer, angiogenesis, along with an inflammatory infiltrate composed of helper T cells, dendritic cells, neutrophils in microabscesses and macrophages.[21] It was argued that these plaque-like lesions were similar to those seen in psoriasis in humans. This was notable because the topical use of imiquimod in patients has been associated with exacerbation of psoriasis.

Some compounds such as pyrethroid insecticides which are employed as topical therapeutic agents for the treatment of skin infestations, produce an irritant response in human skin without morphological changes. This is probably also a pharmacological effect on cutaneous sensory nerve terminals. Such reactions are not detected in conventional animal skin irritation tests.[56]

CONTACT DERMATITIS

Allergic contact dermatitis following exposure to low molecular weight chemicals is distinct from typical primary irritant dermatitis because its development is based on

immunological mechanisms that require an initial sensitizing exposure to the precipitating agent. The reaction is mediated by T lymphocytes and requires penetration of allergen, binding to skin protein to form an antigen and involvement of Langerhans or other antigen presenting cells. The presented antigen reacts with specifically sensitized T cells with production of lymphokines and recruitment of further effecter cells to produce an inflammatory response. Contact dermatitis is typically characterized by a delayed response (24–96 hours) to a patch test containing a non-irritating concentration of the agent.[57]

Preclinical testing for contact allergens has generally employed outbred guinea pigs but mouse sensitization assays are also used.[57] High concentrations of test substance are repeatedly applied to the skin or other technical maneuvers are used to enhance the penetration of allergen. The guinea pig maximization test employs complete Freund's adjuvant in order to potentiate the reaction and detect weak contact allergens.[58]

Results from these protocols are not always predictive for contact allergenicity in humans, particularly for weak sensitizing chemicals that are also primary irritants. As the end result of an immune-mediated inflammatory skin reaction is non-specific inflammation, histopathological examination using routine techniques is not considered particularly helpful in making the distinction between primary irritant and contact dermatitis. However, immunohistochemical techniques using markers for Langerhans cells and subpopulations of T cells may be useful in the characterization of immune-mediated skin reactions in animal models as they have proved to be in the histopathological evaluation of inflammatory skin conditions and contact dermatitis in people.[59] Immunocytochemical study has shown that in the human skin, contact dermatitis is characterized by an infiltrate of mature helper T cells mixture with Langerhans cells.[60]

PHOTO-TOXICITY AND PHOTO-ALLERGY

A variety of drugs cause phototoxic or photo-allergic reactions when they are present in sufficient amounts in the skin. Phototoxic disorders appear to have a higher incidence than photo-allergic conditions and the action spectra for most photo-allergens and phototoxins are in the ultraviolet A range.[61]

A number of *in vivo* and *in vitro* tests have been devised for preclinical testing of photoallergic potential, although there are few standardized methods and the experimental variables are quite diverse.[62] The *in vitro* 3T3 NRU photo-toxicity test is currently widely accepted and is part of an OECD guideline.[63] However, preclinical tests for photosensitization, such as tests for contact sensitization,[64] have not been subject to validation procedures.

The guinea pig and hairless mouse models have been quite widely used, each employing visual assessment of the irradiated skin or measurement of the test skin thickness with vernier skin fold calipers rather than histopathological examination.[65] The auricular skin of albino Balb/Crj (Balb/c) mice has also been used for the histological assessment of the phototoxic lesions induced by quinolone antibacterial agents. Results appear to correlate better with reported phototoxic reactions in patients than those of the *in vitro* 3T3 NRU assay.[66,67]

Kimura and colleagues have proposed that a hairless, pigmented dog is a better model for humans in the investigation of skin toxicity in the context of ultraviolet light irradiation.[68] Histologically, changes of acute phototoxic damage are those of a non-specific

inflammatory response with activation of melanocytes and melanin pigmentation in pigmented species.

Inflammation and ulceration induced by systemic drug administration

Some systemically administered therapeutic agents are capable of inducing inflammatory alterations in the skin of humans and animals. The antiproliferative anticancer drug bleomycin is one well-known example although numerous chemotherapeutic drugs both old and new have been associated with significant dermatological toxicity.[12] Skin lesions produced by anticancer drugs range from mild non-specific exanthematous reactions to those that are dose-limiting.

Cutaneous inflammation and proliferation of epidermal cells has occurred in patients and experimental animals given cytokines such as IL-3, granulocyte and granulocyte–monocyte colony stimulating factors.[69,70] Monoclonal antibodies against the epidermal growth factor receptor (EGFR) or EGFR tyrosine kinase inhibitors are also linked to inflammatory dermatological side effects such as acneiform eruptions, eczema, fissures, telangiectasia and paronychia with pyogenic granulomas in both animals and humans.[71]

Loss of nails (*onychoptosis*) associated with desquamation, erosion or ulceration of the foot pads has been reported in beagle dogs treated with therapeutic agents such as bleomycin which possess a radiomimetic-like effect on squamous mucosa. The antibiotic bleomycin, a mixture of glycopeptides isolated from *Streptomyces verticillus*, possesses antineoplastic activity against squamous cell neoplasms probably as a result of interference with mitosis and inhibition of DNA synthesis.[72] It is believed to be concentrated in the lung and skin because of lower activity of enzymes that inactivate bleomycin in these tissues. Bleomycin is well known for its pulmonary toxicity (see Respiratory Tract, Chapter 6) as well as cutaneous toxicity in humans. Skin changes in humans include hyperpigmentation, induration and nodule formation on the skin of the hands characterized by epidermal acanthosis and focal cellular atypia which can be followed by gangrene.[73]

When administered to beagle dogs, bleomycin produces footpad ulceration. Epithelial lesions commence as alopecia and dermatitis of the tail tip and footpad desquamation. This is followed by ulceration, loss of nails, decubital ulcers and stomatitis.[74] The lesions occur on average after about 40 days of treatment but may develop as soon as one week or following periods as long as 13 weeks after initiation of treatment. The onset of skin lesions is earlier and more severe at high doses.[75] The severity of the lesions is also influenced by the degree of physical trauma on the feet and tail tip. Footpad ulceration is much less severe if dogs are housed on solid plastic floors rather than wire grid floors.[74] The tail tip lesions also appear to result from trauma associated with tail wagging in the confined space of wire grid cages. *Fibrosis* of the dermis or *scleroderma* is the principal change reported in rats rather than ulceration.[76]

Similar nail loss and footpad erosions have been also reported in beagle dogs following administration of high doses of synthetic antiviral nucleoside analogues, BW134U and acyclovir.[77,78] These lesions also occurred between a few days to four or five weeks following initiation of treatment. These footpad lesions were characterized by a defect in maturation of the basal cell layer of the squamous epithelium of the footpads and claw beds and by loss of polarity of the basal cells. The basal cells contained large hypochromatic nuclei and

showed ballooning of the cell cytoplasm. The keratin layer became disrupted with development of erosions, ulcers and nail loss accompanied by active chronic inflammation.

It has been postulated that these drugs affect squamous cell maturation as a result of a direct interaction with cellular components such as DNA or keratin proteins.[74] When such changes coexist with the normal weight bearing and low-grade trauma on the paws, foot ulceration and nail loss result.[78]

In the assessment of the relevance of such lesions for use in humans it is important to assess tissue exposure levels occurring in the affected animals relative to those likely to be achieved in humans. For instance, extremely high concentrations of acyclovir achieved locally at injection site in patients have produced vesicular skin eruptions although under normal clinical circumstances, it appears that insufficiently high local concentrations are achieved to produce skin damage.[79,80] By contrast, bleomycin attains high concentrations in human skin at the doses usually employed in cancer treatment and is consequently associated with significant skin toxicity.

Cytokines and drugs altering growth factors may also produce skin damage. The dermis, connective and parenchymal tissues of rats were shown to develop an infiltration of lymphocytes and eosinophils following intravenous or intraperitoneal injection of high doses of purified human recombinant interleukin 2.[81] The eosinophilic infiltration induced in interleukin 2-treated rats is believed to be secondary to an eosinophilic cytokine produced by interleukin 2-stimulated lymphocytes (see Respiratory Tract, Chapter 6). Disruption of epidermal growth factor receptor (EGFR) tyrosine kinase can also produce inflammation in the skin associated with epidermal proliferation in both experimental animals and humans. The inflammation seems particularly intense around the hair follicles and sebaceous glands on the face and nose in laboratory animals (Figure 2.2). Focal hyperplasia of the epidermis and new hair follicles may also develop in affected areas.[19] The pattern of change in animals appears to mirror that reported in patients treated with these agents. Patients show acneiform eruptions on areas rich in sebaceous glands, notably the face, neck, shoulders, upper trunk and scalp suggesting that the hair follicular is the primary target.[4,71]

Certain inhibitors of cholesterol synthesis provide an example of another class of compounds capable of producing inflammation in the skin. It was shown that two novel aminopyrimidine molecules which inhibited oxidosqualine cyclase produce folliculitis and hair damage associated with epidermal hyperkeratosis and acanthosis of the skin particularly around the ears and eyelids in dogs. It was suggested that the changes were linked to inhibition of cholesterol synthesis because the changes were reminiscent of those reported with triparanol in humans and U18666A in rats and other late stage inhibitors of cholesterol synthesis.[82] This appears to be a class effect related to mode of action for similar findings that have been reported in dogs and hamsters with three other agents of the same class.[83]

Unrelieved vasoconstriction produced by systemic administration of high doses of ergot derivatives can give rise to the necrosis of the tails of rats, the margins of the external ears in dogs and rabbits as well as produce ischemic changes in the peripheral parts of the limbs in humans.[84] Superficial epithelial necrosis of dependent ear margins is also reported in dogs treated for prolonged periods with the ergot compound, bromocriptine.[85]

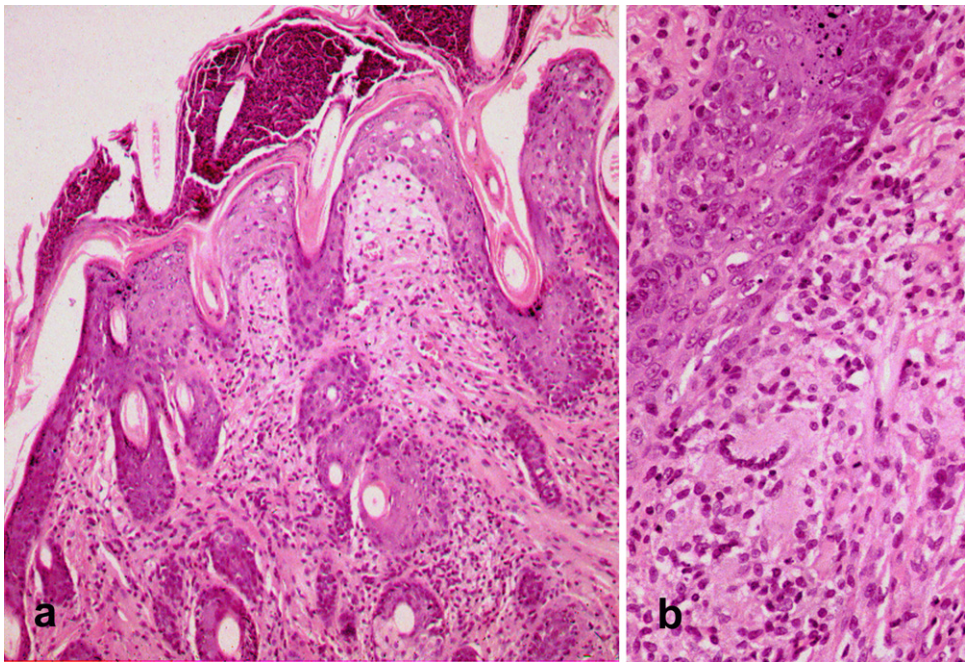

FIGURE 2.2 Skin from the face of a Wistar rat treated with a drug that inhibited epithelial growth factor. *Panel a:* Active inflammation involving the epidermis, dermis and hair follicles. The intact epidermis shows marked irregular reactive hyperplasia or acanthosis (H&E ×110). *Panel b:* Higher power view of the granulomatous reaction within the dermis (H&E ×280).

Skin – changes in pigmentation, hyperpigmentation and hypopigmentation

HYPERPIGMENTATION

Increased pigmentation (*hyperpigmentation*) of the human skin results from treatment with a wide range of systemically administered drugs, including antimalarials, tetracyclines, heavy metals, cancer chemotherapeutic drugs, hormones, phenothiazines and carotenoids.[86,87] Some agents such as corticotrophin, oral contraceptive agents, estrogens, hydantoin derivatives and cytotoxic drugs appear to stimulate melanogenesis either by a direct effect on melanocytes or by mediation through pituitary peptide hormones. Typically, corticotrophin and melanocyte stimulating hormone (MSH) produce a diffuse pigmentation accentuated in light-exposed areas but also with involvement of the oral mucosa, whereas estrogen-induced pigmentation affects primarily the sex hormone-dependent skin over the mammary glands, genitalia and linea alba.[88]

Prostaglandin analogs, currently the most commonly used intraocular pressure-lowering drugs in glaucoma, have been associated with an irreversible change in the pigmentation of the melanin-containing tissues close to the application site on the eyelid skin, eyelashes and iris. This is due to an enlargement of the existing melanin granule population and is of a purely cosmetic effect with few serious consequences.[89,90]

Other substances such as chloroquine, chlorpromazine, β carotene, gold and silver salts and minocycline produce skin pigmentation through the local development of drug−pigment complexes without increasing melanin deposition. Patients treated for long periods with phenothiazines may develop skin pigmentation in sun-exposed areas as a result of lipofuscin-like pigments accumulating in the upper dermis.[91] Not only does minocycline produce pigmentation of the thyroid gland in patients but also more rarely a blue-black discoloration of the skin in those receiving long-term therapy.[92−94] This appears to be the result of an accumulation of iron-containing, electron-dense cytoplasmic granules in macrophages and monocytes of the upper dermis, somewhat similar to the pigment granules reported in the thyroid gland of patients treated with minocycline (see thyroid gland in Chapter 13). Discolored grey skin can be produced in people by administration of colloidal silver products through deposition of metal in the skin.[95,96]

A number of chemicals are reported to cause discoloration of the skin and hair when administered to dogs and rodents in toxicity studies, although correlation between these effects and those in humans is incomplete. Orange discoloration of the fur is reported in albino Sprague-Dawley rats treated with high doses of β carotene.[97,98] Increased melanin deposition was reported in the hormonal-responsive skin of dogs treated with the dopaminergic and prolactin-inhibiting agent, bromocriptine.[85] Brown discoloration of the perianal fur and steel blue coloration of the hairless skin of uncertain significance was reported in albino Wistar rats but not Swiss mice treated for up to two years with β blocker, levobunolol.[99]

HYPOPIGMENTATION

A range of chemicals are used topically in patients to diminish pigmentation in disorders where there is a pathological increase in skin pigments.[100] These include phenolic agents notably hydroquinone derivatives and non-phenolic agents such as azelaic acid and tretinoin.[101] Disconcerting is the widespread topical use of mercury-containing creams in dark skinned young women, particularly in Saudi Arabia, for decreasing the natural pigmentation of the skin. It appears that although this is effective, significant amounts of mercury may be absorbed.[102]

A number of systemic therapeutic agents such as fluphenazine, chloroquine, or corticosteroids may rarely produce *hypopigmentation* of the skin or hair in human subjects particularly when high local tissue drug concentrations are achieved.[88,103,104] Hypopigmentation can result from a reduction in the number of melanocytes, decreased synthesis of melanosomes or incomplete melanin formation. Postulated mechanisms include cytotoxicity, interaction with enzymes involved in melanin synthesis and oxidation of melanin. Grey pallor of the hair has also been noted in hamsters treated with oxidosqualene cyclase inhibitors of cholesterol metabolism in association with other skin alterations.[83] A striking example of a skin color loss in dogs and rats is that produced by an investigational inhibitor of platelet aggregation PD-89454. Treatment of Long-Evans rats for four weeks produced loss of pigment in the cranial pigmented hair. Loss of pigment was also observed in the skin of the nose, around the mouth and eyes as well as the oral mucous membrane in beagle dogs after treatment for four weeks.[105,106] By contrast the skin of pigmented mice was unaffected by treatment.

Histological examination of the affected skin in rats revealed a reduction or loss of pigment in the hair follicle and hair matrix (skin being pigment free in this strain) and loss or lessening of pigment in the basal layer of the skin in dogs. The decrease in pigment was confirmed with Masson-Fontana stain. In both species the DOPA (dihydroxyphenylalanine) reaction was reduced in affected zones. Electron microscopy of the affected skin in dogs showed that melanocytes contained fewer, smaller and incompletely pigmented melanosomes.[106] The exact mechanism was unclear but the dimethoxy substitution of the phenyl ring of this compound suggested that it may have inhibited tyrosinase, an enzyme associated with melanin biosynthesis.[105]

Another example is provided by the greying of the dark hair reported in Long-Evans rats treated for two years with the antihypertensive agent medroxalol hydrochloride.[107] It was postulated that this change related to the binding of medroxalol to melanin because autoradiographic study showed uptake of labeled medroxalol by melanin-containing tissues.

The selective cytotoxicity of some phenolic compounds to melanocytes in pigmented laboratory rodents has been proposed as a rational basis for their application in melanoma chemotheraphy.[75,108] The subcutaneous administration of 4-S-cysteaminylphenol to C57BL/6J mice produced localized de-pigmentation of hair associated with histological evidence of swelling, lysis and necrosis of melanocytes in black hair follicles whereas no degenerative changes were noted in hair follicles of A/J albino mice when administered the same agent.[108] It was postulated that this agent mediated its melanocyte toxicity by interference with melanin synthesis.

Epidermal atrophy

In humans atrophy of the skin is a well-known side effect of prolonged corticosteroid therapy either following systemic administration or topical application.[109–111] Similar atrophy occurs in rodents or pigs following administration of ACTH, systemic or topical application of corticosteroids.[112–115]

These changes following corticosteroid administration appear primarily related to potency and duration of administration. Changes can occur within days in humans and factors such as body site and age influence the degree of atrophy and its reversibility.[110,113] Studies of normal human skin following topical application of a potent corticosteroid (clobetasol-13-propionate) for three weeks have shown that epidermal atrophy is accompanied by compaction of the papillary dermis.[111] After six weeks, the atrophy was shown to be more marked and the changes also involved the deeper reticular dermis. The dermis was also shown to lose collagen and glycosaminoglycan. Fibroblasts became smaller, more ovoid with reduction in cytoplasmic mass and the mast cell population was diminished.[111]

Cortiocosteroid-induced thinning of the epidermis in humans is typically characterized by loss of the granular layer, flattening of the epidermal–dermal border, the presence of pyknotic nuclei in the basal layers and tendency for clusters of epidermal cells to appear above the normal plane of the granular layer.[114] Reductions in the number of Fc-rosetting, C3b-rosetting and Ia antigen-bearing Langerhans cells have been shown to occur in a dose-related fashion, to some extent dependent on the specific corticosteroid employed.[116]

Similar histological alterations occur in the skin of animals following topical administration of corticosteroids. Studies in the domestic pig given topical corticosteroids for seven

weeks showed loss of the granular layer and flattening of the epidermis although the dermis was less affected in this model than in humans.[115]

Our understanding of the mechanisms involved in steroid-induced skin atrophy is incomplete. Corticosteroids have been shown to be capable of lowering epidermal cell mitotic rate, lowering dermal collagen content, decreasing mean diameter of collagen fibrils and decreasing fibroblast cell growth as well as collagen biosynthesis.[110]

Bleomycin also produces atrophy of the skin in rats in association with subcutaneous fibrosis. Rats given bleomycin for periods of up to one year develop skin pigmentation and thickening which becomes evident at three months. These features are characterized by *atrophy* of the epidermis and sebaceous glands accompanied by *fibrosis* characterized by increased numbers of fibroblasts and collagen fibers in the dermis.[76]

Alopecia

A wide variety of drugs are capable of causing damage to hair in humans either directly or indirectly as a result of generalized skin toxicity or systemic disease. Drugs include cytotoxic drugs, colchicine, retinoids, interferons, lithium, heparin, coumarins, some β adrenergic blockers and androgens.[117] Sex hormones, notably androgens, are capable of modulating hair cycling to produce hair loss.[26] Cytotoxic drugs typically induce an abrupt cessation of mitotic activity of anagen and hair shedding within days (*anagen effluvium*). So-called *telogen effluvium* occurs following a premature resting phase (telogen) and hairs are shed much later as club hairs.[117]

Hair loss in laboratory animals also occurs in association with skin lesions induced by systemic administration or local application of drugs or chemicals. It may also be the result of viral or bacterial infection affecting the skin, or in association with infestation with ectoparasites. Hair loss also results from grooming activity particularly in certain strains of mice when housed together. This type of alopecia is limited to grooming regions, most frequently the head but also shoulders, back and pelvic regions. This fur chewing is often preceded by whisker trimming.[118] These behavioral patterns appear to be partly genetically determined in mice for they are highly strain dependent. Nevertheless, whisker trimming and fur chewing may be potentiated in rodents by administration of therapeutic agents, particularly those with activity on the central nervous system. Behavioral-associated alopecia is characterized histologically by hair loss, hyperkeratosis and acanthosis of the epidermis, keratotic plugs in the hair follicles with a mild inflammatory reaction and foreign body type granulomas in the dermis.[119] In pigmented strains, melanin pigmentation may be scattered in the deep dermis and regenerated hair in black mice may be grey in color.

Models of spontaneous androgenetic alopecia seem to be limited to primates where it is reported notably in the stump-tailed macaque (*Macaca arctoides*).[120]

The wasting syndrome in marmosets (*Callithrix jacchus*), a disorder of uncertain etiology characterized by weight loss, muscle wasting, anemia, thrombocytopenia, hypoproteinemia, elevated aspartate aminotransferase and alkaline phosphatase, is also associated with alopecia, particularly of the tail, presumably as a result of the generalized systemic disturbance.[121]

Drug-induced hair loss has been observed in toxicity studies with a wide range of therapies, particularly anticancer drugs and those causing generalized skin damage such as bleomycin.[75]

Hair loss in rodents occurs after dosing with sex hormones or their modulators. Progressive hair loss has been observed in female Wistar rats treated with high doses of a progestogen–estrogen combination.[122] Hair loss was initially observed at the base of the tail and over the lumbar region and progressed cranially and ventrally until complete alopecia was observed after 50 weeks of treatment. The alopecia appeared irreversible even following withdrawal of treatment for 30 weeks. Hair loss or impaired hair growth was also observed in the chronic toxicity studies performed in both rats and dogs with bromocriptine, an ergot analog which inhibits prolactin secretion.[85] Although the mechanism is obscure, it is possible that these effects were the result of prolactin inhibition. Hair loss is reported in humans treated with bromocriptine although clear evidence that it is definitely caused by treatment is lacking.[85] Administration of tamoxifen to neonatal rats has also been reported to adversely affect hair follicles.[123]

Increased hair growth

Cyclosporin A stimulates hair growth in nude mice, possibly by inducing a temporary keratinization of hair in the abnormally keratinizing hair follicles in this strain.[124] Cyclosporin may also produce excessive hair growth in 50% of transplant patients most marked on the face and upper back.[117] A number of other drugs can produce hair growth in unusual areas in patients including minoxidil, phenytoin and diazoxide.[117] Note that this is termed *hypertrichosis*, which is excessive hair on areas other than those affected by androgens, whereas *hirsutism* is excessive growth of coarse hair of male pattern in a female.

Changes in sebaceous glands

A number of drugs and hormones are capable of modulating sebaceous gland activity and morphology. This may become evident in toxicity studies by alterations to the normal silky appearance of the pelage of laboratory animals.

Study of the effects of drugs and hormones on sebaceous gland activity has been conducted in some detail using the hamster flank organ or pilosebaceous units located on the ventral aspect of the hamster pinna.[125,126] Following castration, the large sebaceous glands of the flank organ show atrophy characterized initially by degeneration of sebaceous cells leaving a rim of intact cells at the edge of the gland. Six weeks after castration the glands resemble small sebaceous glands found in normal hamster skin.[126] In view of this sensitivity to androgens, the hamster flank organ can be used to characterize androgen or antiandrogenic activity of novel drugs.[127]

Administration of antiandrogens leads to similar alterations in the flank organ or in the large sebaceous units of the hamster pinna.

Sebaceous glands decrease in size and labeling index in a dose-related manner in hamsters treated with spironolactone.[128] Conversely, administration of testosterone to immature castrated female or even intact male hamsters has been shown to increase the size and pigmentation of the flank organ.[126] Hair loss following anticancer therapy may be partly a result of treatment-induced atrophy or loss of sebaceous glands.[129] Retinoids also cause atrophy of the hamster flank organ and their activity in the gland appears to correlate with their therapeutic effects on acne in humans.[126]

Epidermal hyperplasia

Hyperplasia of the epidermis is observed in laboratory animals and humans as a response to a variety of insults including spontaneous or induced inflammatory processes, application of irritant or toxic substances, repeated abrasion of the superficial stratum corneum and prolonged exposure to ultraviolet light.

It also occurs as a direct response to administered trophic factors. Not surprisingly, administration of epidermal growth factor, a polypeptide which stimulates DNA synthesis and proliferation in epithelial tissues, has been shown to produce a simple epidermal hyperplasia when administered to laboratory animals.[130] As an endocrine-responsive tissue, skin thickening also occurs as a response to growth hormone and somatotrophins. In acromegaly in humans where there is growth hormone excess, much of the thickening is a result of dermal connective tissue proliferation accompanied by increase in coarse body hair and size and function of sebaceous and sweat glands.[24] The effect of growth hormone has been studied in detail in normal beagle dogs. The increase in skin thickness which becomes heavily folded particularly over the forehead and face is also mostly due to an increase in the thickness of dermal collagen.[131]

The histological changes of epidermal hyperplasia are to a certain extent dictated by the nature and severity of inciting stimulus as well as its duration. Features include varying degrees of hyperkeratosis, parakeratosis, prominence of the granular cell layer, increase in the thickness of the squamous cell layer which, when marked, may be characterized by acanthosis and papillomatosis (Figure 2.1). These latter features can be quite florid without being pre-neoplastic in nature.

Hyperplasia also occurs as a response to the topical application of carcinogens. This is usually manifest during the course of neoplastic progression. It is typically associated with atypical cellular features such as nuclear and cellular pleomorphism, excessive or disordered mitotic activity, abnormal keratinization (dyskeratosis) and loss of the normal maturation pattern. In both humans and laboratory animals loss of normal maturation is associated with neoplastic progression. Nevertheless at an early stage it is difficult to make a distinction on histological grounds between a simple reactive hyperplasia or hyperplasia that develops following promotion alone from that which occurs following both initiation and promotion. It has been suggested that a morphometric approach can be helpful in distinguishing various types of nuclear alterations in skin hyperplasia induced in mice by irritant and carcinogenic substances.[132] In a study of the effects of a number of carcinogenic and non-carcinogenic mineral oils on the skin of mice, Ingram and Grasso showed that nuclear enlargement in the epidermis correlated with carcinogenicity in long-term studies.[133] They suggested that morphometric analysis of nuclear size might be useful in discriminating carcinogens from non-carcinogens in the skin.

Induced subcutaneous inflammation

INJECTION SITE INFLAMMATION

Inflammatory changes may be produced in the subcutaneous tissues by substances intended for parenteral administration. Although frank skin necrosis from extravasation of intravenous material into soft tissues is an uncommon complication of therapy in adults, it has been reported in children following infusion of electrolyte solutions containing

potassium and calcium salts, 10% dextrose solutions, vasopressors, radiological dyes, methylene blue and chemotherapeutic agents.[134]

A number of animal models are used for the assessment of local irritant effects of drugs where they are injected subcutaneously and tissues subjected to histopathological analysis. Histopathological examination of the administration sites used in the routine parenteral toxicity studies can be effective for the assessment of the local irritant effects of therapeutic agents. Both the intensity and the nature of the local inflammatory response can be assessed as well as regional effects occurring in the proximal vasculature and in local lymphoid tissue. Ability of any lesions to fully repair can also be evaluated in a reversibility component of such an experiment. The distribution of oily vehicles from injection sites has also been evaluated in lymph nodes by histological examination.[135] Olive oil used for subcutaneous injections in rat studies may produce inflammation and lipogranulomas not only at injection sites but also in other tissues distant from injection sites (see granuloma below).[136] Inflammation is a ubiquitous reaction induced in tissues by aluminum-containing adjuvants in all species as it is important for the recruitment of antigen-presenting cells and the release of cytokines and other mediators that induce maturation and activation of dendritic cells.[137] This inflammation often has a granulomatous pattern and is more severe when vaccines are injected into the subcutaneous tissue rather than skeletal muscle[138] (see granuloma below).

There is a reasonable correlation between local inflammatory effects induced by injection of drugs in humans and animals, although general clinical tolerance may not relate to the degree of inflammation induced. It has been suggested that animal studies predict injection site reactions with biological products poorly.[139] However, with these agents particular consideration must be given to the specific animal model and species-specific pharmacological and immunological differences that may be manifest in soft tissues.

INFLAMMATION INDUCED BY IMPLANTED BIOMATERIALS

Histological assessment of the tissue response to plastics, other polymeric materials and metals implanted in the soft tissues in rodents, rabbits or other species is an important part of the safety assessment and testing of biocompatibility of substances destined for medical applications for which there will be direct contact with human tissues.[140,141] A variety of biocompatibility issues have to be considered and basic schemes for testing are provided in the *International Standards Organization* (ISO) 10993 standards.[142]

The range of animal species used for this assessment is diverse and includes dogs, sheep, pigs and monkeys. However, the choice is important for it depends on the nature, the size and use of the implant and proposed implantation site. Implants have become increasingly complex comprising more than one type of material and incorporating biologically active substances.[143]

Test materials are implanted into the relevant soft tissues for varying lengths of time using appropriate control materials. The tissue reaction is assessed using standard histological techniques. One of the most popular tests for irritancy of a biomaterial is intramuscular implantation in rabbits or rats (see Musculoskeletal System, Chapter 5) and the subcutaneous implantation site can also be used in these species. Intraperitoneal implantation can be used but it may not give such a reliable prediction of tissue reactivity in humans.[144]

Various methods of histopathological evaluation have been employed, but most employ a semi-quantitative assessment of the various components of the tissue response.[145] The amount of necrosis, the character and intensity of inflammation, whether polymorphonuclear or lymphocytic, the presence of plasma cells, macrophages and giant cells and the degree of vascularization and fibrosis are assessed in a semi-quantitative manner to arrive at a final score for tissue reactivity.[144] It is important to assess the tissue response at several time points in order to avoid false positive and false negative results.[140] A negative control such as silicone and a positive control substance such as polyvinyl chloride (PVC) are helpful.[146] Electron microscopic examination including scanning microscopy aid the visualization of changes in cells immediately adjacent to implants, notably protein deposition and corrosion products.[141]

Absolute inertness of implanted biomaterials is uncommon but can be seen with some materials such as pure titanium, high purity alumina and certain polymers such as polyethylene of very high molecular weight and density.[141] While some tissue reaction to biomaterials may be desirable, prolonged chronic inflammation with granuloma formation is to be avoided.

Over recent years advances in biomaterials have provided complex controlled release and implantable delivery systems that often use active biological components. These may require additional studies to address immunotoxicity and biological responses. However, histopathological assessment of any abnormal or prolonged inflammatory responses of the tissues to these novel agents remains an important component of this assessment.[147]

While these animal models appear to accurately predict the local tissue inflammatory response to implanted materials in patients, they may be poor predictors of outcomes of therapeutic or cosmetic implantation in clinical practice. For example, in humans it has been shown that implanted biomaterials subjected to stress such as in joint replacements have the potential to degrade or fragment and disseminate with consequent foreign body reactions and inflammation in other organ systems.[148] Animal models appear not to be reliable predictors of capsular contracture that can occur with silicone or saline-filled silicone breast implants in women[149,150] (see Mammary Gland, Chapter 3).

SUBCUTANEOUS AND SOFT TISSUE INFLAMMATION AND FIBROSIS INDUCED BY SYSTEMIC THERAPY

A particular inflammatory and fibroblastic process involving subcutaneous tissues as well as other connective tissue sites has been reported in animals and humans given various matrix metalloproteinase inhibitors. Matrix metalloproteinases (MMP) are a family of zinc- and calcium-dependent proteinases that enable turnover of the extracellular matrix under normal physiological circumstances and in a number of pathological processes.[151] Some carcinomas overexpress these enzymes leading to breakdown in connective tissue and possibly enhancement of invasion, factors that have led to their clinical investigation for the treatment of cancer.

Toxicity studies with a number of these agents in rats, dogs and primates have shown a fairly consistent pattern of an inflammatory infiltration and fibrosis or a fibroblastic process in connective tissues.[152–155] This process may involve not only subcutaneous tissue and adipose tissue but also connective tissue in muscle, joints and tendons (see Musculoskeletal System, Chapter 5). A detailed study in dogs of the effects of a novel

inhibitor of MMP2, MMP8, MMP9, MMP12 and MMP13 by Westwood and colleagues showed a time- and dose-dependent and extensive fibroblastic process involving many connective tissue sites including subcutaneous tissue, synovium, tendons and skeletal muscle.[152] Microscopically, the changes consisted of monomorphic sheets and bands of cellular connective tissue containing proliferating fibroblasts and myofibroblasts as shown by staining for α-smooth muscle actin. In addition, in some zones a sparse mixed inflammatory infiltrate composed of round cells, a few polymorphonuclear cells and edema was reported. These histological features appear common to other compounds of the same type tested in rats and monkeys although tissue beds affected and severity are variable.

Musculoskeletal side effects are reported in patients treated with these drugs suggesting that similar pathology occurs in response to treatment with these agents. Frozen shoulder and Dupuytren's contracture, a palmar fibroblastic condition composed of proliferating fibroblasts and myofibroblasts, have been reported to occur in patients treated with marimastat, a broad spectrum matrix metalloproteinase inhibitor.[156–158]

Granuloma and granulomatous inflammation

A granuloma is a localized form of inflammation showing an accumulation of histiocytes sometimes accompanied by a sparse infiltrate of polymorphonuclear leukocytes, fibroblasts and proliferating blood vessels. A typical granuloma has a central zone of necrosis surrounded by epithelioid histiocytes that is surrounded by lymphoid cells and monocytes. The term *granulomatous inflammation* is used when there are extensive infiltrates of predominantly histiocytes and macrophages. A minor granulomatous reaction may be seen as a component of many inflammatory processes where there is release of free lipid into the tissues (Figure 2.2). Granulomas not only form as a local reaction to foreign materials but also more widely in soft tissue in response to infectious agents or as an expression of altered function of cells of the monocyte/macrophage series.

In all species the inflammation induced in tissues by aluminum-containing adjuvants often has a granulomatous pattern.[138] Persistent inflammatory nodules called *aluminum granulomas* have been described at injection sites following vaccination of humans and animals.[159,160] These lesions show a diverse and sometimes florid pattern of histological changes including a mixed inflammatory infiltrate, granuloma formation, local fibrosis and fat necrosis. A feature common to all is the presence of histiocytes with violaceous granular cytoplasm as a result of the accumulation of aluminum contained in the vaccine adjuvant.[159]

A lipogranulomatous form of inflammation may develop at the site of injection of oil-based vehicles. This can occur in animals following injection with vaccines containing lipid adjuvants.[160] Olive oil used for subcutaneous injections in rat studies was shown to produce lipogranulomas characterized by the presence of variably shaped clear droplets, unilocular or multilocular macrophages, some lymphocytes and plasma cells and a sparse fibrotic reaction. These lesions were not only found at injection sites but also in distant regions including visceral peritoneum over the mesentery and liver.[136]

It should not be forgotten that injected drugs may inadvertently contain particulate matter that may also produce granulomatous reactions at both local and distant sites. This effect can be seen in people who choose to inject themselves with crushed oral prescription medicines containing filler substances such as talc, corn starch and cellulose.[161]

Some systemically administered drugs have been shown to elicit granulomas or granulomatous inflammation in the soft tissues in toxicology studies as a result of interference with macrophage function. One example was ICI 185,282, a thromboxane receptor antagonist which when administered to beagle dogs produced granulomas in many organs including the skin and subcutaneous tissues.[162] *In vitro* studies showed that ICI 185,282 was able to enhance the migration and accumulation of peripheral monocytes.

Adipose tissue inflammation, fat necrosis and steatitis

Fat necrosis is another form of inflammation that is often visible to the naked eye as white foci in adipose tissue. Histologically, overt necrosis may not be evident but foci of inflammatory cells including macrophages and giant cells are generally present. Clefts left by dissolved cholesterol crystals also occur. Fibroblasts, blood vessels and other connective tissue cells have reactive alterations that can, when exaggerated, give rise to lesions with pseudosarcomatous features.

A generalized form of fat necrosis termed *steatitis* has also been described in rat adipose tissue. It develops in association with vitamin E or antioxidant deficiency that follows excess dietary polyunsaturated fatty acids of the type found in fish or linseed oils.[163] It is characterized by the presence of widely distributed small yellow foci in fat which are composed of clusters of macrophages containing small lipid vacuoles and lipofuscin pigment.

A low-grade chronic inflammatory process in adipose tissue is a well-described characteristic in obesity in both humans and laboratory animals. This is characterized by abnormal cytokine production which has been associated with detrimental metabolic alterations notably insulin resistance and cardiovascular disease.[164–166] It has been shown that obesity causes an increase in activated macrophages and induction of genes such as TNF-α and inducible nitric oxide synthase.[167] Studies in obese mice have shown that histologically, fat contains small interstitial foci of round cells and macrophages, sometimes containing fat droplets or lipofuscin pigment and which stain with macrophage-specific antigen F4/80 (Figure 2.3).[168,169] Interestingly, rosiglitazone, a thiazolidinedione and a peroxisome proliferator-activated receptor (PPAR) γ agonist has been shown to suppress the increased expression of the inflammation genes in the adipose tissue of obese mice.[168]

Other changes in adipose tissue

A relative decrease in the amount of white fat and an increase in brown fat was reported in mice treated with troglitazone. This is a thiazolidinedione drug targeting the peroxisome proliferator-activated receptor (PPAR) γ which is expressed most abundantly in adipose tissue and modifies the cellular response to insulin through enhancement of hepatic glucose utilization and glycolysis.[170] Lipocytes showed increased cytoplasmic eosinophilia and coalescence of cytoplasmic lipid vacuoles. This was associated with increases in BrdU labeling of brown fat cells, interstitial and capillary endothelial cells. It was suggested that this effect might be related to drug-induced effects on nuclear PPARγ and to the resultant up-regulation of the uncoupling protein (UCP-1) in brown fat which enhances the differentiation of preadipocytes to mature brown adipocytes. However, an effect on white and brown fat is reported with a number of other PPARγ agonists in rats, mice and monkeys.[171–173] A two-year study in Fischer-344 rats with the γ-dominant PPAR α/γ agonist naveglitazar also showed histological changes in both brown and white fat.

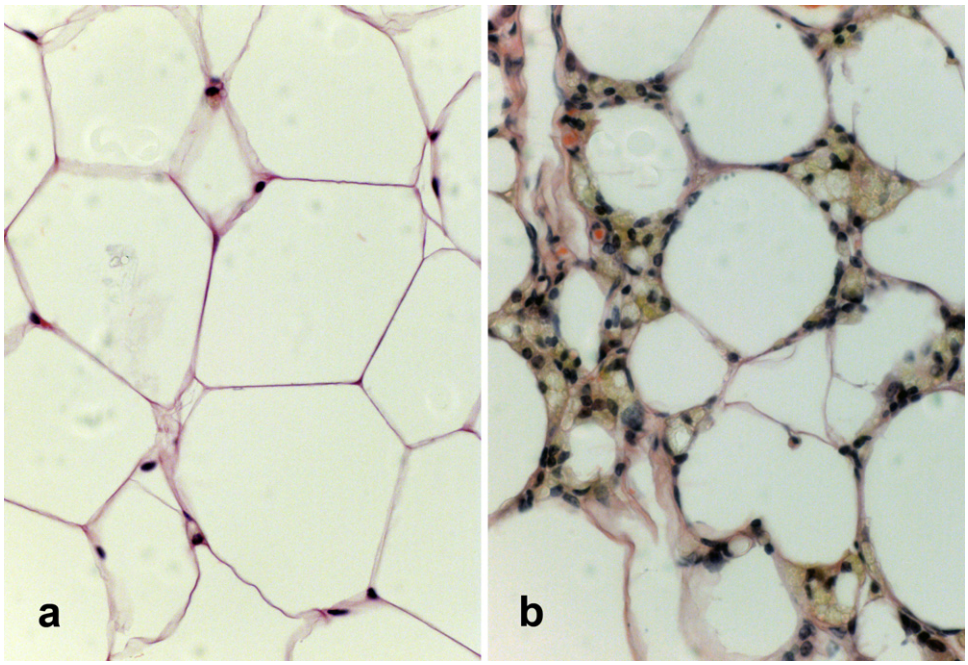

FIGURE 2.3 Abdominal fat from C57BL/6J mice given low fat (10 kcal%) and high fat (60 kcal%) diets. *Panel a:* Normal fat from mouse fed a low fat diet for 17 months. *Panel b:* Fatty tissue from an obese mouse fed high fat diet for 17 months showing a sparse interstitial round cell infiltrate, foamy macrophages and lipofuscin pigment (H&E ×360).

These were described as an increase in droplet size in brown fat and a decrease in size in droplets in white fat. However, in this long-term study there was also an increase in number of undifferentiated mesenchymal cells and intercellular and interlobular matrix.[174]

Brown fat is under the control of the sympathetic nervous system so it can also be stimulated by prolonged exposure to cold, severe hypoxia and following administration of sympathomimetic agents such as norepinephrine (noradrenalin), isoprenaline (isoproterenol) or β_3 adrenergic agonists.[175–177] Studies in a transgenic mouse model have suggested that the mineralocorticoid receptor is also important in brown fat differentiation and regulation of thermogenesis.[178]

ATROPHY OF SUBCUTANEOUS ADIPOSE TISSUE

Administration of recombinant human leptin, a 16 kDa protein that regulates adiposity and body weight, was shown to produce *atrophy of white and brown fat* when administered to C57BL/6 mice for periods of up to 15 days.[179] This was characterized by loss of fat stores with both white and brown fat cells showing depleted cytoplasmic lipid. Brown fat cells became intensely eosinophilic and both brown fat and white fat cells contained numerous large mitochondria. It was suggested that the findings were linked to increased

thermogenesis and lipid oxidation in brown fat and increased lipolysis and decreased fat synthesis in both types of fat.

A well-described adverse effect of protease inhibitors in patients infected with the human immunodeficiency virus is focal loss of adipose tissue (lipodystrophy). Partial lipodystrophy also occurs in patients with endogenous or exogenous long-term corticoid excess.[180] Protease inhibitors may inhibit adipocyte differentiation, induce apoptosis of adipocytes or cause dysregulation of transcription factors involved in adipogenesis.[181,182]

Extramedullary hematopoiesis

Inflammation at injection sites needs to be distinguished from injection site extramedullary hematopoiesis. Cynomolgus monkeys given recombinant human interleukin 3, a hematopoietic growth factor, developed small firm nodules at the subcutaneous injection sites. These nodules contained immature cells of myeloid, erythroid and megakaryocytic series that extended from the subcutaneous tissues into the deep dermis and surrounding adnexa. Eosinophil precursors were the most common with cells of the megakaryocytic series being also prominent in the lesions. Mild fibrosis, neovascularization, edema, perivascular extravasation of blood cells, and at high doses collagen degeneration alongside degenerating eosinophils, were also described.[70]

Elastosis

Solar elastosis is found in sun-damaged skin and is associated with solar keratosis and squamous carcinoma in humans and in sun-exposed skin in animals.[183] It can also be induced in the skin of rats and mice by chronic exposure to artificial ultraviolet light.[184,185] Histologically, it is characterized by accumulation of thickened basophilic staining elastic fibers in the upper dermis of treated animals.

Penicillamine, used in the treatment of Wilson's disease, increases the amount of soluble collagen and induces alteration to elastic fibers in patients. Lesions are characterized histologically by 'lumpy bumpy' or 'bramble bush' protrusions perpendicular to the long axis of elastic fibers. These features correlate with changes in skin fragility and clinical features similar to those found in pseudoxanthoma elasticum.[186]

Amyloid

Deposits of amyloid may be found in the dermis where they are characterized by the presence of pale eosinophilic material. Mice are the most commonly affected. The deposits are stained by Congo red and have an apple-green dichroism when viewed in polarizing light. Sometimes, dermal deposits are associated with subepidermal edema when there is significant systemic amyloid.[187]

Mineralization

Although mineralization is most liable to occur in organs such as the kidney, stomach mucosa, large arteries and myocardium, mineral deposits are sometimes observed in the subcutaneous and soft tissues. In rats, this occurs spontaneously under circumstances which favor generalized mineralization such as a high dietary calcium:phosphate ratio and treatment with substances such as dihydrotachysterol which mobilize calcium stores.[188] This form of mineralization is characterized histologically by fine or coarse

grains of calcium in the dermis and subcutaneous tissue associated with the presence of foreign body giant cells, histiocytes, lymphocytes, fibroblasts and fibrosis. When the deposits become massive, skin ulceration can occur. Zones most affected in rats are trauma sites around shoulders and legs and in mammary tissues in breeding females.

Neoplasms of epidermal origin

Skin cancer can develop following the local application or systemic administration of drugs and other chemicals and excessive exposure to ultraviolet light in both humans and experimental animals.

It is well known that squamous carcinomas occur in the skin of people exposed to polycyclic hydrocarbons for long periods. There is also considerable evidence that each of the three main types of skin cancer in humans, basal cell carcinoma, squamous cell carcinoma and melanoma can be a result of excessive exposure to sunlight. The incidence of each type is higher in fair- rather than dark-skinned people and the risk increases with increasing ambient solar radiation. Squamous carcinoma tends to be associated with occupational exposure and non-occupational or recreational sun exposure is linked mainly to basal cell carcinoma and melanoma.[189]

Cutaneous cancer has also been described in humans following the systemic administration of methoxsalen (8-methoxypsoralen or psoralen) which is used with ultraviolet light A (PUVA) in the treatment of severe psoriasis and cutaneous T-cell lymphoma.[190–193] This drug is administered orally but is photo-activated by exposure of the diseased skin to ultraviolet A which transforms the inert drug to a transiently excited state in which it is capable of covalently cross-linking DNA to achieve a therapeutic effect.[194] Whereas this procedure avoids toxicity to internal organs, low-grade cutaneous epithelial neoplasms are associated with this therapy.[191,192,195,196] High levels of ultraviolet B exposure also increase the risk of a skin cancer in psoralen- and ultraviolet A-treated patients.[197]

The role of the immune system in the inhibition of skin malignancy is reflected by the increased incidence of skin neoplasia in patients receiving immunosuppressive therapy.[14,186] The skin cancer associated with immunosuppression differs from the idiopathic skin carcinoma in the normal ratio of squamous to basal cell carcinomas (1:4) and is reversed in transplant patients.[14]

A vast body of experimental data on the effect of polycyclic hydrocarbons on skin has been developed since the first demonstration of squamous cancer on the skin of rabbits painted with carcinogenic tar in 1918.[198] Cutaneous application of powerful carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA) followed by promoters, typically 12-0-tetradecanoylphorbol-13-acetate (TPA), have been used to study the initiation and promotion sequence in the skin of mice for many years (Figure 2.1). The effects of the initiation–promotion sequence seem to be similar across most laboratory animal species for it has been observed in the skin of rabbits, rats and hamsters.[199–202] However, using DMBA followed by promotion with croton oil, Stenbäck demonstrated the existence of considerable species and strain differences in sensitivity to the development of skin neoplasia.[199] Squamous neoplasms developed readily in Swiss, strain A, Balb/c and C57B1 mice, New Zealand and outbred rabbits, but with difficulty in AKR mice and minipigs.

Hairless strains of mice, the most sensitive being the SKH-2 mouse, have been used to model the tumor development process in response to ultraviolet light.[203] In the SKH-2 hairless mouse reported changes in response to ultraviolet light include epidermal hyperplasia, squamous papillomas, keratoacanthoma-like tumors, skin appendage and basal tumors, actinic keratoses/carcinomas *in situ*, and squamous carcinomas.[204] This model has also been used to study the photo-carcinogenic potential of therapeutic agents such as the quinolone antibiotics.[205] The effects of psoralens have also been studied in the hairless mouse.[203] For example, inflammation, hyperplasia and epithelial atypia were reported in a 13-week toxicity study in which hairless mice were given 8-methoxypsoralen and ultraviolet A radiation in a manner similar to that used in human therapy.[206] The effect of immunosuppression has been modeled in experimental animals, for example in the mouse skin initiated with DMBA and promoted by TPA.[207]

Another mouse model for the study of skin cancer is the Tg.AC transgenic which has an activated *Ha-ras* transgene and requires only promotion with agents such as TPA to form papillomas.[208,209] The hyperplasia and squamous tumors that develop in this model are histologically identical to those occurring in mice after initiation with DMBA and promotion with TPA.[210] Other models include the RasH2 transgenic mouse and the SENCAR mouse, derived with an increased sensitivity to DMBA and TPA promotion.[211]

Only small numbers of skin tumors develop spontaneously in rodents although their incidence increases in older animals. Zwicker and colleagues[212] have reviewed the incidence of skin neoplasms arising spontaneously in aging Sprague-Dawley, Fischer 344 and Wistar rats, Sommer[213] in rats of the Long-Evans strain and Haseman and colleagues in Fischer 344 rats and B6C3F1 mice.[214]

SAFETY ASSESSMENT

A number of reviews of the *National Toxicology Program* (NTP) database have shown that the epidermis along with Zymbal's gland, a modified sebaceous gland in the outer ear, tend to be targets for potent genotoxic carcinogens.[215–217] The NTP data suggest that agents that produce tumors in Zymbal's gland are strongly associated with tumor development not only in the skin but also preputial glands in males and clitoral and mammary glands in females. These tumors are far less commonly induced in rodents by non-genotoxic therapeutic agents.[218] Exceptions are drugs that possess genotoxic activity. These may produce tumors in the skin or at multiple sites including skin. For example, lobucavir, a nucleoside analog produced squamous skin tumors but also squamous cell neoplasms in other organs as well as Harderian gland adenomas and adenocarcinomas in a mouse carcinogenicity study.[219] Nucleoside analogs used in the treatment of viral infections are believed to produce DNA damage through incorporation of the compound into host DNA. Painting mechlorethamine, a genotoxic antineoplastic nitrogen mustard, on the skin of mice for periods up to 33 weeks also resulted in squamous cell skin tumors.[220]

Classification and diagnosis of skin tumors

Neoplasms of epidermal origin can be divided into two main groups for the purpose of safety assessment, (1) tumors of the surface epidermis; and (2) tumors of the epidermal appendages.

A wide variety of different tumor types have been described under these general headings in humans, particularly those showing various types of differentiation towards components of epithelial appendages. Not all of these tumor types have been described in domestic animals, although skin neoplasms are common in domestic species particularly dogs.[221,222] Tumor subtypes showing a variety of epithelial differentiation patterns are observed in aged rodents but they have been generally even less well categorized. Therefore, in rodent safety studies where tumors of similar histogenesis are often grouped for statistical analysis, it is prudent to use a fairly simple classification. Relatively simple classifications have been agreed internationally for use in rodent carcinogenicity studies.[223]

Re-evaluation of skin lesions in Long-Evans rats and comparisons between Sprague-Dawley, Fischer 344 and Wistar rats by Sommer showed that incidences of different skin neoplasms among these were comparable.[213]

A challenge in diagnosis is making the distinction between epidermal hyperplasia, benign neoplasia and invasive carcinoma. This can be difficult in skin that is altered by inflammation or is ulcerated for long periods because reactive changes in the epithelium may develop a pseudo-carcinomatous appearance. Hyperplastic changes in the deep parts of hair follicles may also mimic invasion of cancer cells. As in many tumor systems, evaluation of the non-neoplastic alterations, which precede or are associated with the development of neoplasia, can give important clues to pathogenesis. Such findings need to be assessed in the context of genetic toxicity, pharmacology, disposition data and the intended clinical indications for the drug.

Squamous papilloma

These neoplasms are superficial papillary or pedunculated neoplasms characterized by irregular infolded squamous epithelium showing marked acanthosis, papillomatosis and hyperkeratosis and with a fibrovascular core. They show no evidence of infiltration or invasion of the underlying connection tissues. These lesions occur sporadically in aged untreated rats,[224] mice[225] and hamsters.[226]

Skin papillomas also occur in rats, mice and hamsters following local application of powerful carcinogens where they are believed to arise from the glabrous epithelium rather than from the hair follicle (Figure 2.4).[227] Similar lesions occur in Tg.AC transgenic mice after application of the promoting agent TPA.[210]

Squamous papillomas occur commonly in the skin of dogs where they may be caused by a virus from the papilloma virus group different from the virus that transmits canine oral papillomatosis. Histologically, these canine papillomas contain clusters of cells in the stratum granulosum characterized by clear cytoplasm and eosinophilic intranuclear inclusions. Although there are many different papilloma viruses, common antigenic determinants allow immunocytochemical demonstration of papilloma viruses in different species including those of the dog using the same antibodies.[228] A hamster papilloma virus has been detected in skin papillomas in certain hamster colonies.[226]

Sebaceous adenoma

These neoplasms are composed of proliferating masses of epithelial cells that show a close morphological resemblance to sebaceous glands. They remain sharply localized and

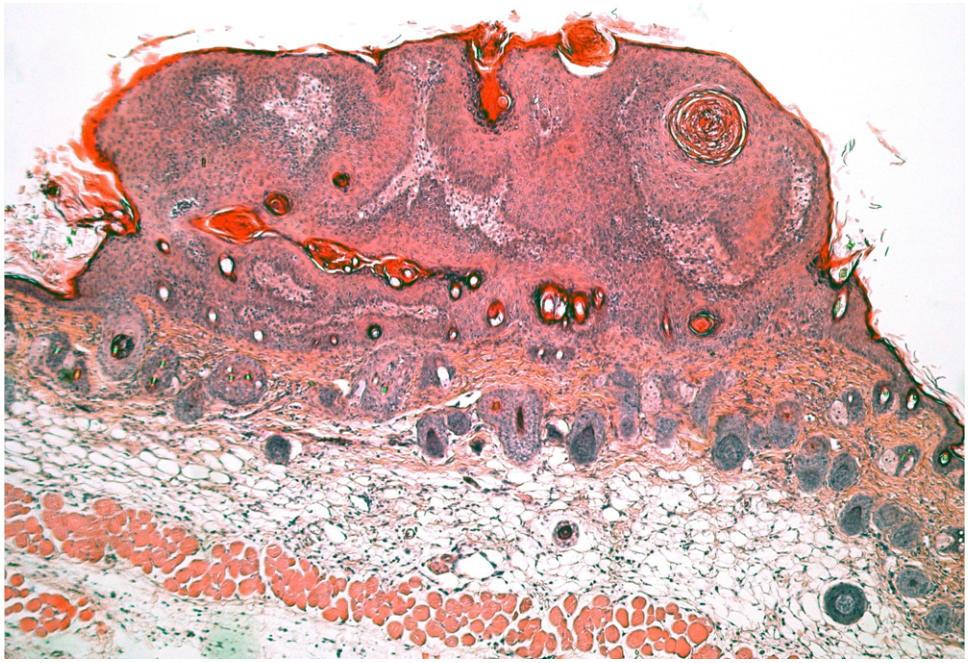

FIGURE 2.4 Well-differentiated squamous papilloma from the back of an FVB/N mouse which developed following the topical application of DMBA and TPA for 15 weeks (H&E ×50).

do not infiltrate the underlying tissues, although cystic change and squamous metaplasia may be present. They are found occasionally in untreated rats.[213,224] They have also been induced in the hamster by topical administration of carcinogens.[202]

Tumors showing hair follicle differentiation

KERATOACANTHOMA

Histopathological study of cutaneous neoplasms induced in rabbits, mice, rats and hamsters by local application of carcinogens led Ghadially to delineate a group of distinctive squamous neoplasms which are morphologically similar to keratoacanthomas in humans.[227] These appear to develop from the hair follicle in contrast to squamous papillomas which develop from the superficial glabrous epithelium. He argued that the superficial cup-shaped lesions developed from the superficial part of the hair follicle whereas the deeper situated rounded cystic lesions arise from the lower part of the hair follicle or hair germ. It has also been shown that the hair follicle may be particularly predisposed to the effects of topical carcinogens because they retain carcinogen for longer periods. These old experiments demonstrate that hair follicles in the telogen phase appear to retain carcinoma for longer periods than follicles in anagen.[227] More recently it has been suggested that the highly proliferative bulge cells of the upper part of the hair follicle possess a long life-span which may predispose them to tumor development on account of accumulation of genetic damage from chemical carcinogens.[27]

Histologically, these experimental keratoacanthomas are characterized by well-defined cup- or bud-shaped proliferation of basal and squamous epithelial cells with a central crater-like mass or cystic formations of excessive, whorled keratin.

Many of the experimental keratoacanthomas appear to grow and regress like hair follicles themselves and can be considered benign neoplasms.[227] Studies of regression in transplantation experiments have suggested that regression has its origin in the hair follicle and is not an immune-mediated phenomenon.[229] Similar tumors are sporadically seen in untreated rats, mice and hamsters.[213,225,230]

Other neoplasms of the skin showing aspects of hair follicular differentiation have been reported as trichoepitheliomas or pilomatrixomas in mice, rats and hamsters.[202,224,225,231] However, not all of these lesions fall into distinct categories so mixed forms are often seen. For example, the otherwise typical squamous papilloma may also show localized hair follicle or keratoacanthoma-type differentiation.

Carcinoma

Carcinomas of the skin present a variety of different histological appearances and can be grouped according to the principal cell type, i.e. basal cell carcinoma, squamous cell carcinoma or sebaceous carcinoma.

Basal cell carcinomas are common skin tumors in humans but much less common in laboratory rodents, including the hairless mouse strains exposed to ultraviolet radiation.[204] Typical basal cell tumors are composed of cells with large, oval or elongated nuclei with poorly defined cytoplasm that resemble basal cells of the epidermis. They are often arranged in masses of various shapes with a palisade arrangement of tumor cells.

Squamous carcinomas are the most prevalent form of skin tumor in rodent skin treated with carcinogen or irradiated with ultraviolet light. They are invasive tumors consisting of irregular penetrating masses of epidermal cells showing varying proportions of normal-looking squamous cells and more atypical, pleomorphic or anaplastic forms. Some malignant epithelial tumors show differentiation towards hair follicles, although such neoplasms are usually all classified as squamous carcinomas. Squamous carcinoma cells may also be particularly pleomorphic and show individual keratinization or spindle cell differentiation resembling mesenchymal cells. It has been shown that some of the spindle cell tumors that occur in the skin of transgenic Tg.AC mice which carry the *v-Ha-ras* transgene are poorly differentiated carcinomas because they contain cytokeratins and desmosomes.[232]

Another pattern of differentiation is the so-called *sebaceous squamous carcinoma*, arising in the rat, usually in the auditory sebaceous (Zymbal's) gland. These are composed of proliferating irregular masses or cords of squamous, sebaceous or basal cells showing variable degrees of mitotic activity and cellular pleomorphism. Single cells, groups or cords of cells penetrate into the dermis and invade deeper tissues. Eventually, there may be involvement of local lymph nodes and distant organs.

Cutaneous carcinomas are only found sporadically in aged untreated rats, mice and hamsters in carcinogenicity bioassays but they have been induced in all three species as well as rabbits by the cutaneous application of carcinogens and promoting agents.[199,200,202] Hairless mice are particularly predisposed to develop carcinomas showing epidermal appendage differentiation in response to ultraviolet light.[204]

Squamous carcinomas have been reported in fairly young beagle dogs housed under conditions of high solar radiation. These squamous carcinomas develop in sparsely haired, lightly pigmented ventral body skin, typically associated with solar keratosis.[233] *Solar keratosis* is characterized by hyperkeratosis, parakeratosis, acanthosis and collagenous thickening of the upper dermis. Solar elastosis may also accompany these lesions (see above).

Neoplasms of the melanogenic system

The epidemiologic evidence implicates sun exposure as a major risk factor in melanoma development in humans.[234] DNA damage caused by ultraviolet radiation has a central role in the pathogenesis of these tumors. Unlike the more common squamous and basal cell cancers, which are associated with total cumulative exposure to ultraviolet radiation, melanomas are linked to intense intermittent exposure. Genetic alterations have been identified in melanomas at different sites which suggest that there are distinct molecular pathways to melanoma, each with a unique relationship to exposure to ultraviolet light.[235]

A slight excess of malignant melanomas has been reported in patients on immunosuppressive therapy.[236] An increased number of benign nevi have also been reported in children on cancer chemotherapy.[237] Methoxsalen (8-methoxypsoralen or psoralen) used with ultraviolet light A (PUVA) in the treatment of severe psoriasis and cutaneous T-cell lymphoma has also been associated with the development of malignant melanoma which appears after about 15 years from the first treatment particularly among patients who receive 250 treatments or more.[238]

Pigmented strains of rodent occasionally develop neoplasms of melanogenic cells with advancing age so these neoplasms may be found sporadically in carcinogenicity bioassays performed in these strains.[213,225,239–243] Neoplasms of melanin-producing cells are also widespread among certain domestic animals particularly in heavily pigmented species.[222]

Melanomas can also be induced in pigmented rodents such as the hamster and C57BL/6 mice by the cutaneous application of carcinogens.[202,244] Melanomas have also been induced in animals by exposure to ultraviolet radiation.[245,246]

Whereas in human diagnostic pathology the term melanoma is usually reserved for malignant melanoma, in veterinary pathology, the term melanoma has often been more widely employed to embrace various forms of benign neoplasms which are described as nevi in man.[221]

Nevi (benign melanoma)

As in humans, junctional, compound and intradermal nevi are recognized in animals.[221] A *junctional nevus* is one in which clusters or nests of rounded or polygonal melanocytic cells are present at the dermal–epidermal junction. In the *intradermal nevus*, nests or bundles of well-differentiated, rounded melanocytes are located exclusively in the dermis. The so-called *compound nevus* combines features of both junctional and intradermal types.

Intradermal nevi resembling the so-called *blue nevus* described in humans are also found in laboratory animals. This nevus is found in the dermis and is characterized histologically by an ill-defined proliferation of spindle-shaped cells or fibrous melanocytes, usually

laden with melanin pigment. They may be found in pigmented strains of mice, hamsters and rats.[247]

Malignant melanoma

These neoplasms show broadly similar histological patterns to benign nevi but are composed of atypical or pleomorphic cells, which may show marked mitotic activity. They can be composed of cells of epithelioid type which may spread both along the epidermis and into the dermis or be composed of fibrous or spindle cells.

In the hamster both the epithelioid type with junctional activity and the spindle or fibrous cell forms are well described.[248] Spindle cell forms appear to be more commonly described in pigmented strains of rats and mice. Burek described eight malignant melanomas of aged Brown-Norway rats out of a population of 310.[239] Unlike the hooded Long-Evans which possesses pigmented hair, the Brown-Norway rat has heavily pigmented skin and brown hair. Most of these melanomas occurred on the extremities and they invaded local tissues and spread to local lymph nodes. Sommer found only two malignant melanomas in a control population of 980 Long-Evans rats.[213] Ward found only two malignant melanomas in 5,065 pigmented B6C3F1 mice.[225]

In C5BL/6 mice treated with 7,12 dimethylbenz(a)anthracene and croton oil, malignant melanomas were of dermal spindle cell type and there appeared to be a progression from benign nevi similar to human blue nevi, through to premalignant cellular blue nevi.[244]

Over recent years amelanotic melanoma has been increasingly recognized in rats. In Fischer 334/N rats they occur in less than 1% of aged animals and the pinna is a frequent site. They show the cellular features of melanoma but are devoid of pigment. Although they stain for S100 protein, this does not enable distinction from schwannomas because a variety of mesenchymal tumors and normal tissues also contain S100. However, studies with the electron microscope have shown intracytoplasmic premelanosome, single membrane-bound organelles containing membranous filaments.[249,250]

Subcutaneous (soft tissue or mesenchymal) neoplasms

The histopathological diagnosis of soft tissue neoplasms remains one of the more difficult issues in tumor pathology. Usually these neoplasms are found relatively infrequently in routine rodent carcinogenicity bioassays and a simple classification is usually appropriate. Nevertheless, in the context of chronic inflammation of soft tissues, separating reactive proliferating changes from mesenchymal neoplasia can be an extremely difficult diagnostic challenge. Their characterization has much in common with the diagnosis of human soft tissue tumors where the classification used for clinical management is less complicated than the 'scientific' classification based on detailed patterns of tissue differentiation.[251]

Induced subcutaneous soft tissue neoplasms: injected and implanted substances

In rats and mice, subcutaneous administration of powerful carcinogenic chemicals such as polycyclic hydrocarbons, as well as the repeated subcutaneous injection of a range of agents not generally considered carcinogenic, can induce sarcomas around the injection sites after varying periods of time. Agents among the latter class include concentrated

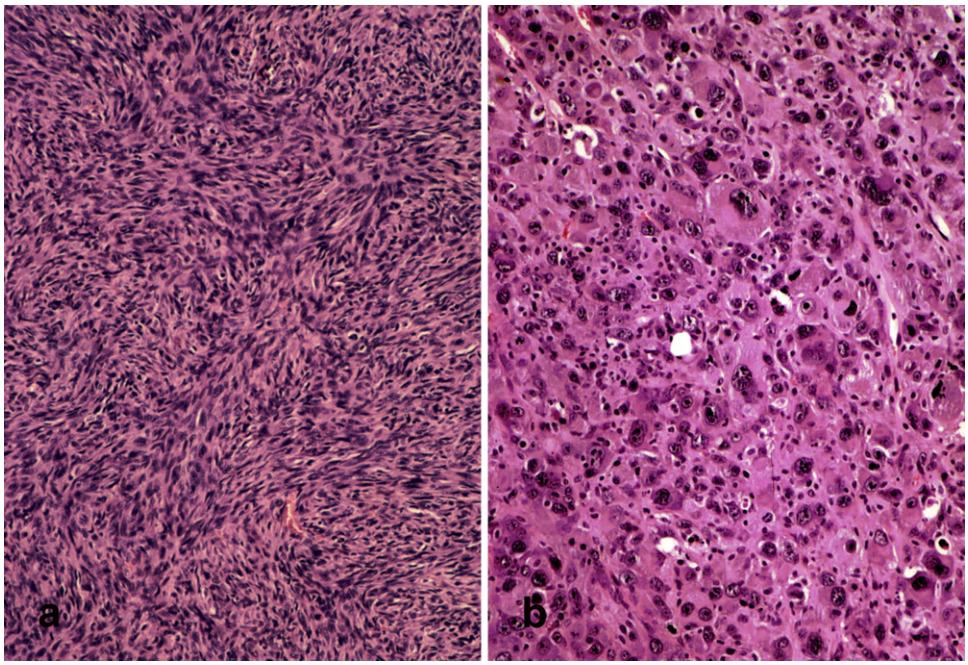

FIGURE 2.5 Sarcomas which developed in Sprague-Dawley rats implanted with millipore filters 12 months previously. *Panel a:* The spindle form with cells arranged in bundles and in a storiform pattern. *Panel b:* A pleomorphic variant containing a large number of giant cells (H&E ×110).

solutions of glucose and other sugars, sodium chloride, certain water-soluble food colorings and surfactants, carboxymethycellulose and macromolecular dextrans.[252–254] Some of these materials such as macromolecular iron dextrans have been used therapeutically in humans by the parenteral route for many years without evidence of tumor induction.[253] Likewise subcutaneous implantation of a variety of substances including inert plastics and other materials used in medical prostheses can give rise to sarcomas around implantation sites in rodents, the so-called *Oppenheimer effect* or *solid state carcinogenesis* (Figure 2.5).[144,255,256] These phenomena remain unexplained and do not fit easily into conventional concepts of tumor initiation, promotion and progression. As a consequence tumors developing at injection and implantation sites of novel therapeutic agents can pose problems of interpretation, particularly as it may be difficult to separate the solid-state effects from the effects of the chemical properties of the injected or implanted substance.

Sequential studies of local tissue reactions to repeated subcutaneous injections of non-carcinogenic substances have tended to show a correlation between the nature of the early lesions and the ultimate formation of sarcomas. It appears that if injected substances do not elicit a massive macrophage response, cause little or no damage and are adequately absorbed from the injection site, neoplasia does not result.[252] By contrast, agents that elicit a response characterized by severe inflammation, tissue damage, a macrophage response, fibroblastic proliferation and fibroplasia tend to be associated with the development of sarcomas.

Early tissue responses observed around implanted inert plastics of dimensions appropriate to produce sarcomas are also characterized by inflammation, monocytic and macrophage response, fibroblastic proliferation and dense fibrosis.[256] Although no clear relationship between the type of tissue response and chemical structure of the injected or implanted material has been clearly discerned, it has been suggested that the pattern of initial tissue response is related to physical characteristics of the material such as surface activity, lipid solubility and protein binding.[253,257] In the case of subcutaneous implants, size, shape and form appear to be the most critical elements in sarcoma development in animal models.[144] A study by Kirkpatrick and colleagues showed that a number of different commercially biomaterials implanted in Fischer 344 rats as smooth discs of identical dimensions and surface characteristics all produced sarcomas at the implantation sites, the earliest appearing after 26 weeks.[258]

The situation with implanted solids parallels the tumorigenic effects of fibrous mineral particles injected into rodents because the effect appears to be dependent on dimensions and durability of fibers rather than their precise chemical structure.[259] This has raised questions about the tumorigenicity of injected nanoparticles. Hansen and colleagues have shown that injection of nanoparticles can induce local sarcomas in rats at injection sites. However, in this study sarcomas developed only with nanoparticles composed of nickel or cobalt but not titanium dioxide, silicone dioxide or polyvinylchloride.[260] This suggests that factors other than dimension may have been important in this study. Nickel and cobalt may have more inflammatory reactivity in nanoparticulate form.[261]

In general the development of sarcomas at injection sites is not considered to indicate potential cancer hazard to humans for non-genotoxic drugs administered by other routes.[257,262] The difficulties in interpretation are usually circumvented by avoiding administration by the subcutaneous route for long-term toxicity studies. However, the increasing requirement to assess the safety of agents by parenteral routes to avoid first pass metabolism or of lack of absorption from the gastrointestinal tract and the need to assess new biomaterials may necessitate consideration of these local effects at injection or implantation sites. In long-term studies in rodents some biodegradable materials remain intact for longer periods than planned giving rise to unexpected development of neoplasms that can be explained on the basis of a *solid-state* effect.

Chronic damage to tissues caused in other ways may also be associated with mesenchymal tumor development. One example is the development of spindle cell neoplasms on the ear tips of rats treated for long periods with ergotamine, at the site of tissue damage produced by this agent.[263]

Established sarcomas which follow injection or implantation of a variety of different agents have usually been classified as fibrosarcomas, malignant fibrous histiocytomas or simply as spindle cell or pleomorphic sarcomas (Figure 2.5). However, fibromas, osteogenic sarcomas, rhabdomyosarcomas, histiocytomas, leiomyosarcomas, angiosarcomas and liposarcomas have all been reported.[253,256,258] More recent studies of these sarcomas in rats have suggested that most of the spindle cell and pleomorphic neoplasms are similar to sarcomas reported in humans.[258,264] There appears to be some differences in tumor types induced by different agents although these are not marked. Roe and Carter suggested that histopathological characteristics of the established tumors arising in rats at the sites of injection of iron dextran were dose dependent.[265] It appeared that the more pleomorphic

tumors developed after high doses of iron dextran whereas lower doses produced more fibrous and spindle cell subtypes.[265,266] In the more recent study by Kirkpatrick and colleagues the histological features of induced sarcomas showed little or no correlation with the biomaterial composition except that angiosarcomas appeared to be slightly more common with polyurethane implants.[258]

In contrast to the tissue responses around inert plastics and the various non-carcinogenic agents, it has been shown that the early tissue response and the latent period before development of sarcomas is different with frankly genotoxic carcinogenic agents. Carcinogens such as N-methyl-N-nitrosourea, 7,12-dimethylbenz(a)anthracene or N-nitroquinoline-N-oxide appear to inhibit connective tissue repair and produce morphologically, atypical or bizarre fibroblasts as early responses.[267,268] Sarcomas develop in rats after a relatively short period of about 20 weeks, in contrast to the 50 weeks or more taken for sarcomas to develop in rats around inert plastic implants and non-carcinogenic chemicals.[267] The sarcomas that eventually develop are generally pleomorphic sarcomas (malignant fibrous histiocytomas) although neonatal administration of 7,12-dimethylbenz(a)anthracene appears to produce more rhabdomyosarcomas.[269] It is worth noting that the genotoxic actinomycin D used as an anticancer agent was also reported to induce sarcomas at local injection sites in mice, although the evidence that this agent is carcinogenic in humans is lacking.[270]

SPECIES DIFFERENCES

There appear to be species differences in the sensitivity to solid-state carcinogenicity. Reported incidences of sarcomas at the site of implanted small glass and polypropylene covered microchips in Fischer rats is about 1% and in mice the range is between about 2 and 4% in B6C3F1 mice, 1.2% in CBA/J female mice, 0.5% in CBA/J male mice whereas CD-1 mice are more resistant.[271–273] Most striking is the development of foreign soft tissue sarcomas around microchips implanted into the subcutaneous tissues of heterozygous transgenic $p53^{+/-}$ mice within periods as little as 15 weeks.[274] These sarcomas show a similar range of histological appearance to those induced by foreign bodies in conventional rodent strains.

The cat appears to have a particular predisposition to soft tissue sarcoma development at the sites of vaccination or after implantation of foreign materials[275–277]. Of the 34 reports received in the United Kingdom of injection site sarcomas in cats in 2005, 23 were associated with live vaccines, nine with inactivated vaccines and one with an ectoparasiticide.[278] The reason for the particular predisposition of the cat is uncertain. However, it has been shown that cats differ in the early local inflammatory response to commercial rabies vaccines compared to other small carnivores such as mink and ferrets and it has been consequently suggested that this might be linked to the predisposition to vaccine-associated sarcomas.[279]

Sporadic reports of sarcoma development around metallic and non-metallic foreign bodies in both human subjects and domestic animals have raised concern that *solid-state carcinogenesis* or the *Oppenheimer effect* may have some relevance to the implantation of medical devices and prostheses. Various histological types of sarcoma including osteosarcoma, fibrosarcoma, malignant fibrous histiocytoma and undifferentiated sarcomas have been sporadically reported in humans around the site of shrapnel fragments and

orthopedic metallic and ceramic prostheses.[280,281] However, following review of these case reports, paying attention to both the nature of the implants and the experimental data on the carcinogenicity of metals in rodent bioassays, Sunderman suggested that this tumorigenic effect may be related to certain metals in the implanted alloys, notably nickel and cobalt under circumstances in which corrosion occurs with release of these metallic ions into the surrounding tissues.[281] Although both industrial and surgical exposure to these two metals cause inflammatory and other immune reactions in tissues directly exposed, and long-term industrial exposure of the lungs to hexavalent chromium is associated with risk of cancer, the development of sarcomas adjacent to solid implants composed of these metals appears rare in humans.[261]

Induced subcutaneous soft tissue neoplasms: drugs administered systemically

Much less common are reports of chemicals that induce soft tissue tumors as a result of systemic administration. Most are genotoxic industrial chemicals which induce subcutaneous tumors along with neoplasms in other organs.[282] Until recently only one or two marketed drugs have been associated with soft tissue tumor development in conventional rat or mouse carcinogenicity bioassays. However, over recent years the peroxisome proliferator activated receptor γ and γ/α agonists studied for the treatment of diabetes have been associated with the development of soft tissue tumors along with tumors in the bladder so this has become considered a class effect of this type of drug.

Peroxisome proliferator-activated receptor γ and γ/α agonists have been associated with the development of fibrosarcomas, lipomas and liposarcomas in rats and hemangiosarcomas in mice.[171–173,283,284] The compounds are not mutagenic. The fibrosarcomas and liposarcomas appear closely associated with an increase in the amount of subcutaneous fat and increases in mesenchymal cell proliferation as shown by BrdU labeling.[171] The vascular tumor response has been linked to the high baseline proliferative rate of endothelial cells in mice compared with that in humans (see below).[285]

Quinapril hydrochloride, an inhibitor of angiotensin-converting enzyme inhibitor, was associated with a slight increase in subcutaneous lipomas along with mesenteric lymph node hemangiomas in female rats given the highest dose in a conventional bioassay.[286] The antifungal drug itraconazole was associated with a slightly increased incidence of soft tissue sarcomas in male rats at three times the maximum recommended human dose. This was thought to be linked to hypercholesterolemia which occurred in rats but not in other species.[287]

Classification

The histopathology of soft tissue neoplasms shows relatively little interspecies variation. One of the traditional differences between humans and rats has been the frequency with which fibrosarcomas are reported. In the rat, it has been considered a common sarcoma with a wide spectrum of different histological appearances ranging from monomorphic spindle cell tumors to highly pleomorphic types.[288] In humans fibrosarcomas are considered fairly uncommon neoplasms of monomorphic spindle cell arranged in interlacing fascicles or herringbone pattern whereas the malignant fibrous histiocytoma has until recently been considered one of the most common soft tissue types of adult life. While this tumor was barely recognized in experimental pathology until recent years, it is now

accepted that many of the sarcomas previously regarded as fibrosarcomas in rats closely resemble what have been called malignant fibrous histiocytomas in humans.[289–291] Similar observations have been made in other species including mouse,[292,293] hamster and dog.[294]

However, over recent years there has been a significant shift in the classification of human soft tissue tumors which is embodied in a consensus classification of the *World Health Organization*.[295,296] One of the major changes following more extensive immunohistochemical and molecular study is that the pleomorphic malignant fibrous histiocytoma does not represent a single entity but a number of different tumor types each of which may have a particular biological behavior and may require different therapy.[297] For example, those showing immunohistochemical or ultrastructural evidence of myogenic differentiation appear to behave in a more aggressive manner.[297,298] Those pleomorphic tumors showing no specific differentiation patterns are now termed *undifferentiated pleomorphic sarcomas* but they are believed to comprise less than 5% of adult soft tissue sarcomas.[296]

Another example of a similarity is the spindle cell pattern found in virus-induced rodent sarcomas. Many years ago this pattern was recognized by Chesterman and colleagues as being similar to Kaposi's sarcoma found in humans.[299] This is particularly pertinent since the observation that Kaposi's sarcoma develops in people affected by the human T cell lymphotrophic virus (acquired immunodeficiency syndrome, AIDS) or in transplant patients where immunosuppression plays an important part in its development and clinical progression.[300,301] It is associated with Kaposi's sarcoma-associated herpes virus or herpesvirus 8. This virus has been detected by the polymerase chain reaction in over 90% of Kaposi's sarcoma lesions.[302] Compelling evidence now shows that this virus is the main factor in the development of this tumor.[303]

The currently accepted standardized nomenclature for rodent soft tissue tumors uses relatively simple categories according to the principal pattern of differentiation as the range of tumors defined in rodents is much less than in humans.[304–307] These classifications proved suitable with only minor modifications for the assessment of the mesenchymal tumors occurring in rats, mice and hamster following treatment with the PPAR agonists.[283]

Histogenesis

Some of the difficulties in the understanding and diagnosis of mesenchymal neoplasms relates to their diverse histological appearances and the considerable overlap observed in morphological features between neoplasms of different types. This has been accentuated more recently by the demonstration of ultrastructural features common to many mesenchymal neoplasms, notably the presence of primitive cells and immunocytochemical demonstration of common antigenic constituents. Such features can be explained by the concept that sarcomas do not develop from mature cells but from pluripotential primitive mesenchymal cells which fail to differentiate or remain arrested in development along one or more of the many possible pathways of cell differentiation.[308,309]

Fibroma

This term is limited to subcutaneous nodules or masses composed of dense interwoven bands of collagen, interspersed with a sparse scattering of small fibroblast-like cells showing little or no cellular pleomorphism or mitotic activity. These lesions are well localized

and usually solid, although focal myxomatous degeneration may be seen. Fibromas are seen in untreated aged rats where they may be difficult to distinguish from mammary fibroadenomas in which atrophy of glandular elements has occurred.

Fibrosarcoma

The diagnosis of fibrosarcoma is applied to monomorphic sarcomas composed of spindle cells with oval nuclei and basophilic cytoplasm arranged in interlacing fascicles or interwoven in a herringbone pattern. These neoplasms show variable mitotic activity that can be intense and there is usually a collagenous intercellular matrix. Other features such as giant cells, the storiform pattern of smooth muscle differentiation, are typically not seen.

The cytoplasm of sarcoma cells shows ultrastructural features usually dominated by rough endoplasmic reticulum either as slender profiles or dilated by moderately electron-dense amorphous material. Characteristically, fibrosarcoma cells contain cytoplasmic intermediate filaments (7–10 nm diameter) of the vimentin type.[310] Thin filaments (4–6 nm diameter) have also been described in rat fibrosarcomas, usually arranged in bundles near the cell membrane, features suggestive of myofibroblast differentiation.[311]

In both animals and humans fibrosarcomas behave as locally invasive neoplasms, spreading widely in skeletal muscle with relatively late and infrequent metastatic spread. They have been recently reported to develop in rats given agonists of the peroxisomal-proliferator-activated receptor of the dual α and γ subtypes although their relevance for humans is uncertain.[172]

Pleomorphic fibrosarcoma, pleomorphic sarcoma, malignant fibrohistiocytoma

Malignant tumors of this group have until recently been considered some of the most commonly occurring soft tissue sarcomas in humans, particularly in older age groups.[312,313] They now fall into several subgroups based on immunohistochemical and electron microscopic characterization of the component cell types.[296–298,314] Those devoid of any evidence of differentiation are now termed *undifferentiated pleomorphic sarcomas.*

Similar neoplasms have been well characterized in the rat where they occur spontaneously.[290] These pleomorphic tumors are the principal type of sarcoma induced in laboratory rodents by implanted chemical and inert substances.[258,264,315–317] They occur spontaneously in mice, hamsters, dogs, cats, horses, pigs, rabbits, cattle and birds.[293,294,318–320] In domestic cats they also seem to be the principal sarcoma type induced at vaccine inoculation sites.[275]

Histological features are variable, ranging from a fairly well-ordered storiform or cartwheel pattern of plump spindle cells to patterns composed of highly pleomorphic mixtures of spindle cells, small rounded cells and multinucleated and bizarre giant cells (Figure 2.5). A mononuclear or polymorphonuclear infiltrate may also be seen and blood vessels may be prominent. Collagen formation is usually marked in spindle cell areas and myxoid change may be seen. Hemorrhage, necrosis and focal accumulation of iron pigment also occur. Giant cells may present strap-like features suggestive of skeletal muscle differentiation. However, cross-striations are not seen and myoglobin is not detected in the tumor cell cytoplasm by immunocytochemical techniques.