Third Edition

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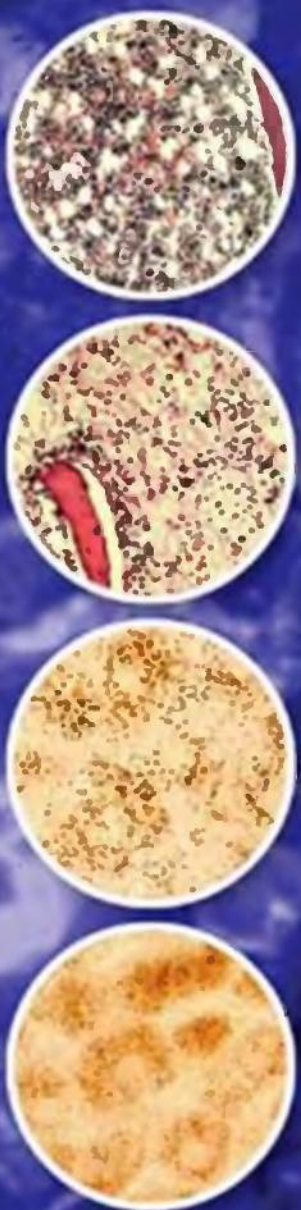
Histopathology of Preclinical Toxicity Studies



Preface

Since the first edition of this book in 1990, there have been many new developments in the treatment of disease. Molecular biology has brought additional understanding to the pathogenesis of a number of diseases. Despite some well-publicized problems with a few drugs because of adverse effects in patients, successful novel therapies have made further significant contributions to treatment of important diseases. However, all new therapies require safety testing in animals before they can be studied in people. Whilst the practice of preclinical drug safety evaluation has retained most of its conventional methodology, the potency and novelty of some of the newer drugs have brought increased complexity to the interpretation of pathological effects in animals and assessment of their relevance or lack of relevance for patients.

In view of this and the persistent gap in the literature relating to pathological changes in preclinical drug safety studies, this book has been updated to include newer classes of therapy whilst retaining the format and style of the previous editions. As before, some old references have been retained, for these contain important studies of drug-induced pathology. Moreover, these references are sometimes not easily located by modern computer search tools.

The outstanding difficulty in this area of drug development remains the prediction of likely adverse effects in patients based on findings in laboratory animals. Unfortunately, reviews comparing drug effects in animals with those in patients remain scanty, particularly when it is considered that this form of experimental study has been practised for well over 50 years.[1] In view of this, decisions relating to the progression or cessation of drug development based on preclinical data are often difficult and sometimes contentious. Decisions need to be taken as early as possible in the life cycle of potential new drugs, often when data is incomplete, so that risks to patients are minimized and resources are not wasted on poor candidate drugs.

In view of the importance of the extrapolation of drug effects in animals to patients, this book also reviews the available data comparing adverse effects produced in toxicity studies with those occurring in humans. This has been done for each organ or organ system. It is hoped that this will provide some help for those taking drug development decisions when faced with drug-induced pathology in toxicity studies.

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Histopathology of Preclinical Toxicity Studies : Interpretation and Relevance in Drug Safety Evaluation

by Greaves, Peter

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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.

Although there have been many changes over the past few decades 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 sulphanilamide over 60 years ago[1] (Table 1.1). The basic paradigm of dosing laboratory animals with various doses of new drug for increasing periods of time accompanied by careful clinical observations, biochemical and haematological monitoring followed by histopathological examination of the tissues remains essentially unaltered and has withstood the test of time. The pathologist is not only required to evaluate alterations to organs and tissues and any relationship that they might have to drug treatment but also to assess the 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 by people of chemicals such as arsenic with those induced by administration to animals.[2] A thorough and systematic review of pathology induced by toxins in humans and animals was published by Orfila as long ago as 1815.[3] Although in the modern era drug safety evaluation has been practised in rodent and non-rodent species widely since before the Second World War, there have been

Table 1.1 Principles of drug testing before trials in humans as defined in 1938 by Geiling and Cannon[1]

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

very few critical comparisons of the effects of drugs in man and these laboratory animals. Much potentially useful information still resides in archives of pharmaceutical companies and government agencies. Nevertheless, the available data suggests that the traditional approach using 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.[4] Indeed, dosing a rodent and non-rodent species with a new drug up to one month identifies over 90% of adverse effects that that will ever be detected in conventional animal studies. However, more generally these studies do not predict all adverse drug effects that can occur in clinical practice and there remains significant over- and under-prediction 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.[4] Animal studies seem to over predict 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 haemodynamic effects.

Treatment-induced findings in conventional toxicity studies found in different laboratory animal species also seem to possess different prognostic value for humans. Although the data is fragmentary, findings in beagle dogs studies appear overall to be better predictors of human adverse effects than data from rodents or, surprisingly, from primates.[4] Dog gastrointestinal and cardiovascular physiology appears to model particularly well for humans.[7,8]

Another long-standing problem highlighted recently 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 their perceived lower adverse effect profile on the gastrointestinal tract compared with conventional drugs but this benefit was outweighed by an increased incidence of cardiovascular disease in some patients. Such effects are difficult if not impossible to predict from 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.[9,10] Such interactions usually require randomized controlled trials specifically designed to look for such risks.[9] 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 toxicity in children.[11] 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 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 medicines for food-producing animals. Whilst 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.[12]

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.[13] 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 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.[14] These studies are quite widely practised but they require careful design, critical selection of models and careful pathology evaluation. 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'. These studies require the careful diagnosis of diverse tumours and so called 'pre-neoplastic' 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.[15] If genotoxic agents are excluded, the majority appear to have induced tumours as a consequence of exaggerated or unwanted pharmacodynamic effects at high doses which have not precluded their use in patients for treatment of disease. As noted by Cohen, the classical model of multistage carcinogenesis of initiation–promotion–progression is no longer adequate to explain many of these effects.[16] Characterisation of genotoxic activity, direct or indirect mitogenesis, cytotoxicity, apoptosis or modification of differentiation is likely to provide a more fruitful avenue for the assessment of carcinogenic hazard for humans.

It has also long been argued that the traditional mouse carcinogenicity study adds little or nothing to the evaluation of carcinogenicity and is consequently a redundant test.[17] Monro has suggested that because of improved understanding of rodent tumorigenesis, that a single study of 12–18 months' duration in rats alone would be sufficient to identify potential human carcinogens.[18] Redundancy of the mouse assay is widely agreed and as a consequence other studies, notably in genetically engineered mice, have been permitted as substitutes by government regulatory authorities. These have proved temperamental studies and have not lived up to expectations so they appear to be no better than conventional assays.[19] Consequently, companies are naturally unwilling to take risks of late stage rejection or delay of major and costly projects by governments through omission of the traditional mouse assay or with problems of interpretation of findings in a study in genetically modified mouse.

Government agencies also recommend that the mouse assay is conducted with classes of compounds such as the novel peroxisome proliferator-activated receptor agonists where there is a perceived problem of carcinogenic potential.[20] Consequently, both conventional rat and mouse carcinogenicity studies 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. Although the results are often due to exaggerated or unwanted pharmacodynamics at high doses of little relevance to patients at therapeutic doses, it remains the role of the pathologist to provide the explanation and indicate likely relevance or lack of relevance for humans.

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.[21,22] 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 an human tumours 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*'.[23]

Lack of critical correlation means that terminology common to laboratory animal and human pathology can mislead. A term used for a rodent lesion may reflect pathology of a quite different biological behaviour in humans. For example, rat mammary carcinomas have a different biological behaviour to the common breast carcinomas in women. Mouse pulmonary tumours 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. For example, basal cell carcinomas of the skin are the most common cancers associated with exposure to ultraviolet light in humans but squamous carcinoma is the principle tumour type induced in animals.[24]

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 that 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.

Pathological techniques

Over the past few years a number of excellent reviews of standardized techniques for use in the histopathology evaluation of toxicology studies have been produced covering tissue selection, blocking and sectioning procedures, immunocytochemical stains for laboratory animals and other basic techniques.[25–29] In addition, the scientific literature and suppliers' catalogues are replete with interesting techniques and novel reagents that can be applied to tissue sections. Some of these can be very useful in the analysis of pathological alterations in toxicity studies, some fail to work in routinely fixed material. However, it is important that these techniques are used in a judicious manner with clear aims following careful analysis of conventional haematoxylin and eosin stained sections. This is particularly true for the application of microarray and bioinformatics technology. Whilst undoubtedly useful in toxicology, these techniques should not be applied in isolation but in combination with other information, particularly pathology.[30]

Above all, there is no substitute for good, conventional histopathological analysis. Unfortunately there remain widespread misconceptions about the nature of the pathological evaluation that lead to demands for additional techniques such as quantitation and 'blinded' slide reading even if these add little to the evaluation. Histopathological examination is not an exercise in 'picture matching'. It represents a careful step by step evaluation of tissue and cellular patterns. This includes assessment of the size, shape, staining characteristics and organisation 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 is in this analysis that special stains can be helpful. Classical histological or histochemical stains are important to confirm the nature of substances such as pigment or cytoplasmic vacuoles. The assessment of numbers of endocrine cells can be enhanced by immunocytochemical staining for the appropriate hormone or receptor. Cell proliferation can be more accurately estimated by use of antibodies to cell-cycle proteins. However, most of these represent an aid to not a substitute for careful histopathology assessment.

Reporting of pathology findings

Report writing represents the final but one of the most important tasks of the pathologist. It requires particular clarity as reports serve 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 specialty. At the other extreme are lay people, for example on ethical review committees, who will have no 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 is essential. The comments of British writer George Orwell, author of *1984*, 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. Whilst 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 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.[3] 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 findings, particularly pathology, based on informed judgement and realistic understanding of the limits of 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 endeavour.

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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 2–3% of hospitalized patients.[1,2] They may be the largest proportion of drug-related causes of emergency department visits and hospital admissions.[3] Non-steroidal anti-inflammatory drugs, the penicillins and trimethaprim-sulphamethoxasole are associated with a particularly high rate of adverse skin reactions. A large proportion of these skin reactions appear to be a result of hypersensitivity or other immune-mediated reactions.[4] Cutaneous drug reactions are particularly common in patients with human immunodeficiency virus (HIV) infection and their incidence increases as immune function deteriorates.[5] Whilst most of these reactions are not severe, some skin reactions, such as toxic epidermal necrolysis or Lyell's syndrome, can be life threatening if treatment is not discontinued.[6] In addition, the incidence of skin carcinomas increases with the duration of immunosuppressive therapy, particularly in white transplant recipients.[7] All this is perhaps not surprising when it is considered that the skin is the largest organ of the body which acts as a physical barrier and has a highly complex defence function capable of considerable resistance to ultraviolet light and a vast variety of external antigens. The skin may be particularly predisposed to drug hypersensitivity reactions because it functions as an immunological organ in which keratinocytes, Langerhans cells and T lymphocytes form an integrated system mediating cutaneous immunosurveillance.[8]

Whilst many immune-mediated drug reactions appear to develop from the response to hapten-carrier complexes, it appears that some cutaneous

immune-mediated drug reactions result from metabolism-independent T cell stimulation through drug binding directly to the MHC-peptide complex on antigen presenting cells and T cell receptors.[9] Activation of T cells seems to lead to a particular clinical picture such as formation of pustules or bullae.[4] In addition, phototoxic and photo-allergic reactions also occur as a result of both systemically or topically administered therapeutic agents.[10,11] However, in many cases it has not been possible to delineate pathogenic mechanisms because the skin only responds with a relatively limited number of patterns to a diverse range of adverse stimuli.

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 such reactions only poorly.[12] 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 Olsen 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.[13]

Different components of the skin, including keratinocytes, dendritic cells, monocytes, lymphocytes, mast cells and vascular endothelial cells, can form the primary target for cutaneous toxicity and may have a role in the determination of the clinical symptoms.[4] 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. Wound healing is modulated by numerous cytokines including transforming growth factors, fibroblast and platelet-derived growth factors, tumour necrosis factor α, interleukin 1, colony stimulating factor 1 and vascular endothelial growth factor.[14] These are potential targets of modern therapies and cutaneous reactions have been reported with some cytokines when used therapeutically in humans.[15] For example, a pharmacologically mediated mechanism has been proposed for the common cutaneous reactions that occur with imatinib mesylate. This drug, which selectively inhibits bcr/abl and other non-specific tyrosine kinases, such as c-kit and platelet derived growth factor (PDGF) receptor is used in the treatment of chronic myeloid leukaemia. It has been suggested that the similarity of the skin reaction to imatinib mesylate to that induced by mercury which also inhibits some tyrosine kinases supports the concept that the adverse effects are related to the pharmacological effect of the drug.[16–18]

The skin also exhibits alterations that are manifestation 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.[19]

Hair follicle

The hair follicle can be a target for therapeutic agents. Although they vary in size and shape depending on location, the basic structure of hair follicles is similar – rapidly proliferating matrix cells in the hair bulb and a hair shaft composed of intermediate filaments and associated proteins. 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.[20] This 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.[20] 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, that have abnormal hair follicle development.[21]

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). 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. However, 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 and permeability constraints. 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 that of most laboratory animals and the pH of the dog is one of the highest of all mammalian species.[22]

On the basis of *in vivo* studies with various labelled chemicals, it has been shown that permeability of animal skin can be ranked in decreasing order of permeability: rabbit, rat, pig and man, with the skin of the miniature pig possessing the closest permeability characteristics to that of human skin.[23] A comparative study of the percutaneous absorption of C_{14} radiolabelled 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

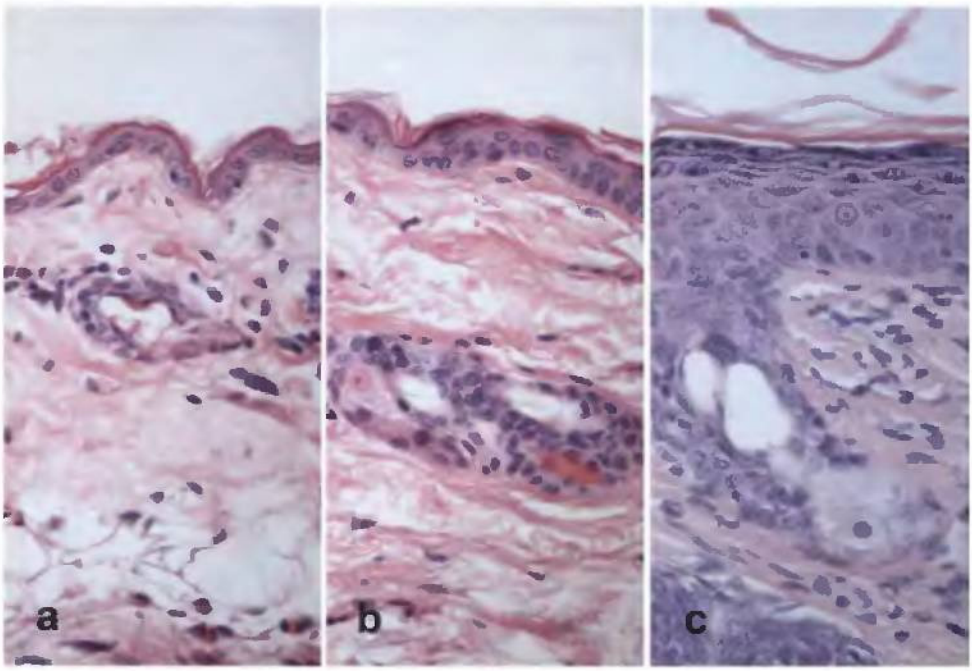

Figure 2.1 *Panel a:* Normal skin from the abdomen of a FVB/N mouse. *Panel b:* Normal skin from the back of a FVB/N mouse. The epidermis is thicker on the back than the abdomen. *Panel c:* Epidermal hyperplasia on the back of a FVB/N mouse as a result of the daily local application of TPA and acetone for two weeks. There is marked reactive hyperplasia of the epidermis, which is thickened. Epidermal cells are enlarged and there is a prominent keratohyalin layer. All at the same magnification (H&E ×250)

a similar rank order in the absorption of the molecules. Although the ratios of absorption between rat and the different sites in man were different, they remained constant.[24] 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.[25] Furthermore, increased exposure of the underlying connective tissue, skeletal muscle and joints to high concentrations of therapeutic substances administered topically may also occur.[26] 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.[27]

The histological pattern and cell types involved in cutaneous delayed hypersensitivity reactions appears to vary among different species.[28] 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.[29] Epidermal Langerhans cell density has also been shown to decrease with advancing age in female BALB/C mice.[30]

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 that 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.[31]

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 haloes, features similar to those seen in the skin of humans or other animals infected with poxviruses.[32]

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.[33] 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[34] 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.

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

Skin irritancy

For topically administered therapy, potentially adverse skin effects are assessed by local application before use in humans. However, the predictive potential of animal models proposed for the assessment of irritancy potential of therapeutic agents remains uncertain and controversial. Despite considerable efforts to identify new *in vitro* methods, none appears to be completely validated.[35] Hence, Draize-type testing using the rabbit and incorporating techniques such as hair shaving, abrading and use of occlusive patches remains widespread.[36] 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.[37]

However, interspecies comparisons of the skin irritancy potential of chemicals have shown that neither the rabbit nor guinea pig skin model is entirely reliable as a predictive model for humans and that there may be a degree of over- or under-prediction, depending on the type or potency of the irritant substances.[38–41] In general terms, most animal models appear capable of predicting compounds that cause severe irritation in humans, but uncertainties remain in the prediction of mild or moderate irritancy potential.[42]

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.[37] 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 probably contribute to distinguishing between different vascular and cellular responses in the early phases of chemically induced skin irritation.

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 a pharmacological effect on cutaneous sensory nerve terminals. Such reactions are not detected in conventional animal skin irritation tests.[43]

Histological changes in skin irritancy studies

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 (see 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. Oedema 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.[44]

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 sensitising 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.[45]

Preclinical testing for contact allergens has generally employed outbred guinea pigs but mouse sensitization assays are also used[45]. High concentrations of test substance are repeatedly applied to the skin or other technical manoeuvres 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.[46]

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 more precise characterization of immune-mediated skin reactions in the various animal models, as they have proved to be in the histopathological evaluation of inflammatory skin conditions and contact dermatitis in people[47]. 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.[48]

Cutaneous phototoxicity

A variety of drugs cause phototoxic or photo-allergic reactions when they are applied to the skin or reach it via the blood stream. A number of *in vivo* and *in vitro* tests have been devised for preclinical testing of photo-allergic potential, although there are no standardized methods and the experimental variables are quite diverse.[10] 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 callipers rather than histopathological examination.[49] 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.[50] Kimura and colleagues have proposed that a hairless, pigmented dog is a better model for humans in the investigation of dermatotoxicity in the context of ultraviolet light irradiation.[51] Histologically, changes of acute phototoxic damage are those of a non-specific inflammatory response with activation of melanocytes and melanin pigmentation in pigmented species.

Injection site inflammation

Inflammatory changes may be produced in the subcutaneous tissues by substances intended for parenteral administration to humans. 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.[52]

Persistent inflammatory nodules called *aluminium granulomas* have also been described at injection sites following vaccination or allergen desensitization

in humans.[53] 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 aluminium contained in the vaccine adjuvant.[53]

Although a number of special animal models are used for the assessment of local irritant effects, 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.[54]

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 for substances destined for medical applications for which there will be direct contact with human tissues.[55,56] The range of animal species used for this assessment is diverse and includes dogs, sheep, pigs and monkeys. However, the choice can be critical for it depends on the nature, the size and use of the implant and proposed implantation site. Increasingly implants incorporate biological active substances which may also influence choice of species particularly if a human protein.[57]

Test materials are implanted into the relevant soft tissues using appropriate control materials for varying lengths of time. 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 used in these species. Intraperitoneal implantation can be used but it may not give such a reliable prediction of tissue reactivity in man.[58]

Various methods of histopathological evaluation have been employed, but most employ a semi-quantitative assessment of the various components of the tissue response. 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.[58] It is important to assess the tissue response at several time points in order to avoid false positive and false negative results.[55] A negative control such as silicone and a positive control substance such as polyvinyl chloride (PVC) are helpful.[59] Electron microscopic examination, including scanning

microscopy, aid the visualization of changes in cells immediately adjacent to implants, notably protein deposition and corrosion products.[56]

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.[56] Whilst 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 more 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 is an important component of this assessment.[60]

It is important to note that whilst 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.[61] Animal models appear not to be reliable predictors of capsular contracture that can occur with silicone or saline-filled silicone breast implants in women[62,63] (see Mammary Gland, Chapter 3).

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 example. More recently, 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.[15,64] Monoclonal antibodies against the epidermal growth factor receptor (EGFR) or EGFR tyrosine kinase inhibitors are also linked to inflammatory dermatological adverse effects such as acneiform eruptions, eczema, fissures, telangiectasia and paronychia with pyogenic granulomas.[65]

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.[66] 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 include hyperpigmentation, induration and nodule formation on the skin of the hands characterized epidermal acanthosis and focal cellular atypia which can be followed by gangrene.[67]

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.[68] 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.[69] 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.[69] 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* seems to be the principle change reported in rats rather than ulceration.[70]

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.[71,72] These lesions also occurred between a few days to 4 or 5 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.

Although the pathogenesis of these lesions is uncertain, 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.[68] When such changes coexist with the normal weight bearing and trauma on the paws, foot ulceration and nail loss results.[72]

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.[73,74] By contrast, bleomycin is believed to attain 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.[75] 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). 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.[65]

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 that inhibited oxidosqualine cyclase produce folliculitis and hair damage associated with epidermal

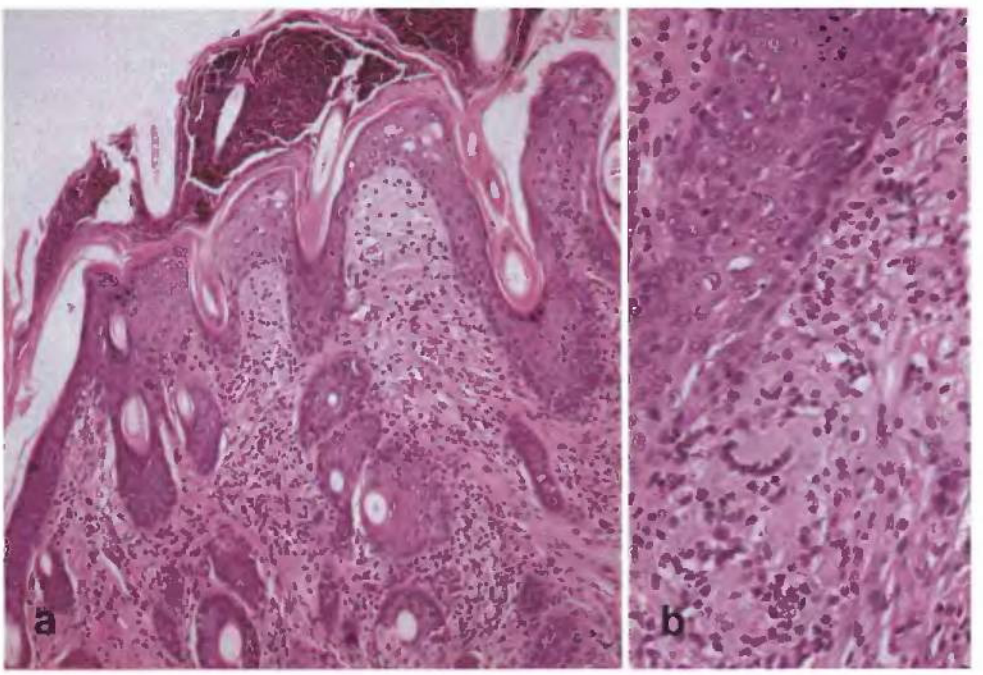

Figure 2.2 Skin from the face of a Wistar rat treated with a drug that inhibited epithelial growth factor. *Panel a:* Active inflammation involving the epidermis and hair follicles which involves the dermis. Although the epidermis is intact it shows marked irregular reactive hyperplasia or acanthosis (H&E ×110). *Panel b:* Higher power view of the granulomatous reaction within the dermis (H&E ×250)

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, other late stage inhibitors of cholesterol synthesis.[76] This appears to be a class effect related to mode of action, for similar findings have been reported in dog and hamster with three other agents of the same class.[77]

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 ischaemic changes in the peripheral parts of the limbs in humans.[78] Superficial epithelial necrosis of dependent ear margins is also reported in dogs treated for prolonged periods with the ergot compound, bromocriptine.[79]

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 leucocytes, 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.

Some systemically administered drugs have been shown to elicit granulomas or granulomatous inflammation in the soft tissues in toxicology studies, presumably 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.[80] *In vitro* studies showed that ICI 185,282 was able to enhance the migration and accumulation of peripheral monocytes.

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.[81] 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.

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.[82] Lipocytes showed increased cytoplasmic eosinophilia and coalescence of cytoplasmic lipid vacuoles. This was associated with increases in BrdU labelling 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, this effect is common to a number of other PPARγ agonists.[83]

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 noradrenalin, isoprenaline (isoproterenol) or β_3-adrenergic agonists.[84–86]

Studies in a transgenic mouse model have suggested that the mineralocorticoid receptor is also important in brown fat differentiation and regulation of thermogenesis.[87]

Extramedullary haematopoiesis

Inflammation at injection sites needs to be distinguished from injection site extramedullary haematopoiesis. Cynomolgus monkeys given recombinant human interleukin 3, a haematopoietic 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,

oedema, perivascular extravasation of blood cells, and at high doses, collagen degeneration alongside degenerating eosinophils, were also described.[64]

Changes in pigmentation, hyperpigmentation, hypopigmentation

Hyperpigmentation

Increased pigmentation (*hyperpigmentation*) of the human skin results from treatment with a number of systemically administered drugs. Some agents such as corticotrophin, oral contraceptive agents, oestrogens, hydantoin derivatives and cytotoxic drugs appear to stimulate melanogenesis either by a direct effect on melanocytes or 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 oestrogen-induced pigmentation affects primarily the sex hormone-dependent skin over the mammary glands, genitalia and linea alba.[88]

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.[89] Not only does minocycline produce pigmentation of the thyroid gland in patients but also rarely a blue–black discolouration of the skin in those receiving long term therapy.[90–92]. 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, Chapter 13). Discoloured grey skin can be produced in people by administration of colloidal silver products through deposition of metal in the skin.[93,94]

A number of chemicals are also reported to cause discolouration of the skin and hair when administered to dogs and rodents in toxicity studies, although correlation between these effects in animals and those in humans is uncertain. An example is the orange discolouration of the fur of albino Sprague–Dawley rats treated with high doses of β carotene.[95,96] Increased melanin deposition was reported in the hormonal-responsive skin of dogs treated with the dopaminergic and prolactin-inhibiting agent, bromocriptine.[79] Brown discolouration of the perianal fur and steel blue colouration of the hairless skin of uncertain significance was reported in albino Wistar rats but not Swiss mice treated for up to 2 years with β blocker, levobunolol.[97]

Hypopigmentation

A range of chemicals are used topically in patient to diminish pigmentation in disorders where there is a pathological increase in skin pigments. These

include phenolic agents, notably hydroquinone derivatives and non-phenolic agents such as azelaic acid and tretinoin.[98] 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 systemically.[99]

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.[88,100,101] 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.[77] A striking example of a skin colour loss in dogs and rats is that produced by an investigational inhibitor of platelet aggregation PD-89454. Treatment of Long–Evans rats for 4 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 4 weeks.[102,103] 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.[103] 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.[102]

Another example is provided by the greying of the dark hair reported in Long–Evans rats treated for 2 years with the antihypertensive agent medroxalol hydrochloride.[104] It was postulated that this change related to the binding of medroxalol to melanin because autoradiographic study showed uptake of labelled 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.[69,105] The subcutaneous administration of 4-S-cysteaminylphenol to C57BL/6J mice produced localized depigmentation 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.[105] It was postulated that this agent mediated its melanocyte toxicity by interference with melanin synthesis.

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.[106] It can also be induced in the skin of rats and mice by chronic exposure to artificial ultraviolet light.[107,108] Histologically, it is characterized by accumulation of thickened basophilic-staining elastic fibres 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 fibres in patients. Lesions are characterized histologically by 'lumpy bumpy' or 'bramble bush' protrusions perpendicular to the long axis of elastic fibres. These features correlate with changes in skin fragility and clinical features similar to those found in pseudoxanthoma elasticum.[109]

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 oedema when there is significant systemic amyloid.[110]

Atrophy

Epidermal atrophy

In humans atrophy of the skin is a well-known adverse effect of prolonged corticosteroid therapy either following systemic administration or topical application.[111–113] Similar occur in rodents or pigs following administration of ACTH, systemic or topical application of corticosteroids.[114–117]

These changes following corticosteroid administration appear primarily related to potency and duration of administration. However, changes can occur within days in humans and factors such as body site and age influence the degree of atrophy and its reversibility.[112,115] Studies of normal human skin following topical application of a potent corticosteroid (clobetasol-13-propionate) for 3 weeks have shown that epidermal atrophy is accompanied by compaction of the papillary dermis.[113] After 6 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 glycosaminoglycans. Fibroblasts became

smaller, more ovoid with reduction in cytoplasmic mass, and the mast cell population diminished.[113]

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 a tendency for clusters of epidermal cells to appear above the normal plane of the granular layer.[116] 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.[118]

Similar histological alterations occur in the skin of animals following topical administration of corticosteroids. Studies in the domestic pig given topical corticosteroids for 7 weeks also showed loss of the granular layer, flattening of the epidermis, although the dermis was less affected in this model than in man.[117]

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.[112]

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 3 months. These features are characterized by *atrophy* of the epidermis and sebaceous glands accompanied by *fibrosis* characterized by increased numbers of fibroblasts and collagen fibres in the dermis.[70]

Atrophy of subcutaneous tissue

Administration of recombinant human leptin, a 16kDa 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.[119] 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). Protease inhibitors may inhibit adipocyte differentiation, induce apoptosis of adipocytes or cause dysregulation of transcription factors involved in adipogenesis.[120,121]

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.[122] Sex hormones, notably androgens, are capable of modulating hair cycling to produce hair loss.[20] 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.[122]

Hair loss in laboratory animals also occurs in association with skin lesions induced by systemic administration or local application of drugs or chemicals, as a 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.[123] These behavioural 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. Behavioural-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.[124] In pigmented strains, melanin pigmentation may be scattered in the deep dermis and regenerated hair in black mice may be grey in colour.

Models of spontaneous androgenetic alopecia seem to be limited to primates, notably the stump-tailed macaque (*Macaca arctoides*).[125]

The wasting syndrome in marmosets (*Callithrix jacchus*), a disorder of uncertain aetiology characterized by weight loss, muscle wasting, anaemia, thrombocytopenia, hypoproteinaemia, elevated aspartate aminotransferase and alkaline phosphatase, is also associated with alopecia, particularly of the tail, presumably as a result of the generalized systemic disturbance.[126]

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.[69]

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–oestrogen combination.[127] 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 analogue which inhibits prolactin secretion.[79] 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.[79] Administration of tamoxifen to neonatal rats has also been reported to adversely affect hair follicles.[128]

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.[129] Cyclosporin may also produce excessive hair growth in 50% of transplant patients, most marked on the face and upper back.[122] A number of other drugs can produce hair growth in unusual areas in patients, including minoxidil, phenytoin and diazoxide.[122] 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 in a male pattern in a female.

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 that favour generalized mineralization such as administration of a high dietary calcium:phosphate ratio and treatment with substances such as dihydrotachysterol which mobilize calcium stores.[130] 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.

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.[131,132] 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.[132] 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 labelling index in a dose-related manner in hamsters treated with spironolactone.[133] 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.[132] 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.[132]

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.[134] As an endocrine-responsive tissue, skin thickening also occurs as a response to growth hormone and somatotrophins. However, in acromegaly in humans where there is also 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.[19] 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 mostly due to an increase in the thickness of dermal collagen.[135]

The histological changes of epidermal hyperplasia are to a certain extent dictated by the nature and severity of the 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. It should be noted that the 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.[136]

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.[137] They suggested that morphometric analysis of nuclear size might be useful in discriminating carcinogens from non-carcinogens in the skin.

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. A vast body of experimental data on the effect of these agents on skin has been developed since the first demonstration of squamous cancer on the skin of rabbits painted with carcinogenic tar in 1918.[138] 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 2.3). The effect of the initiation–promotion sequence seems to be similar across most laboratory animal species for it has been observed in the skin of rabbits, rats and hamsters.[139–142] 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.[139] 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.

There is considerable evidence that each of the three main types of skin cancer in humans, basal cell carcinoma, squamous cell carcinoma and melanoma, is 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.[143] Hairless strains of mice, the most sensitive being the SKH-2 mouse, have been used to model the tumour development process in response to ultraviolet light.[144]

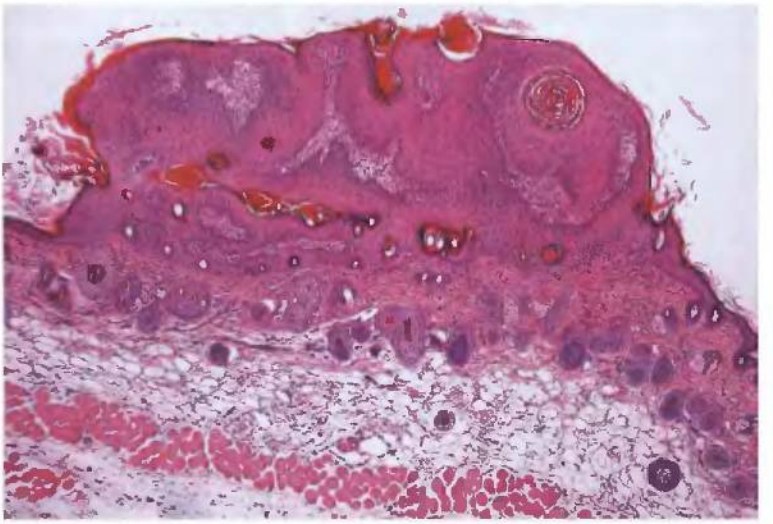

Figure 2.3 Well-differentiated squamous papilloma from the back of a FVB/N mouse which developed following the topical application of DMBA and TPA for 15 weeks (H&E ×35)

In the SKH-2 hairless mouse reported changes in response to ultraviolet light include epidermal hyperplasia, squamous papillomas, keratoacanthoma-like tumours, skin appendage and basal tumours, actinic keratoses/carcinomas *in situ*, and squamous carcinomas.[145] This model has also been used to study the photo-carcinogenic potential of therapeutic agents such as the quinolone antibiotics.[146]

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.[147–149] This drug is administered orally but is photoactivated 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.[150] Whereas this procedure avoids toxicity to internal organs, low-grade cutaneous epithelial neoplasms are associated with this therapy.[148,149,151,152] High levels of ultraviolet B exposure also increases the risk of a skin cancer in psoralen and ultraviolet A-treated patients.[153] These effects have also been studied in laboratory rodents, notably the hairless mouse.[144] 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.[154]

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.[7,109] The skin cancer associated with immunosuppression differs from the idiopathic skin carcinoma in the normal ratio of squamous to basal cell carcinomas (1:4) is reversed in transplant patients.[7] The effect of immunosuppression has been modelled in experimental animals, for example in the mouse skin initiated with DMBA and promoted by TPA.[155]

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.[156,157] However, the hyperplasia and tumours that develop in these models are histologically similar to those occurring in other models.

Only small numbers of skin tumours develop spontaneously in rodents although their incidence increases in older animals. Zwicker and colleagues[158] have reviewed the incidence of skin neoplasms arising spontaneously in ageing Sprague–Dawley, Fischer 344 and Wistar rats, Sommer[159] in rats of the Long–Evans strain and Haseman and colleagues[160] in Fischer 344 rats and B6C3F1 mice.

Classification and diagnosis of skin tumours

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

A wide variety of different tumour 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 tumour types have been described in domestic animals, although skin neoplasms are common in domestic species, particularly in dogs.[161,162] Tumour 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 tumours of similar histogenesis are often grouped for statistical analysis, it is prudent to use a fairly simple classification. 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.[159]

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 tumour systems, evaluation of the non-neoplastic alterations, which precede or are associated with the development of neoplasia can give important clues to pathogenesis. Moreover, 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,[163] mice[164] and hamsters.[165]

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 (see Figure 2.3).[166] Squamous papillomas occur commonly in the skin of dogs where they may be caused by a virus from the papilloma virus group but one that is different from the one 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.[167] A hamster papilloma virus has been detected in skin papillomas in certain hamster colonies.[165]

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; do not infiltrate the underlying tissues, although cystic change and squamous metaplasia may be present. They are found occasionally in untreated rats.[159,163] They have also been induced in the hamster by topical administration of carcinogens.[142]

Tumours 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.[166] 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. Moreover, these old experiments demonstrate that hair follicles in the telogen phase appear to retain carcinoma for longer periods than follicles in anagen.[166] 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.[166] Studies of regression in transplantation experiments have suggested that regression has its origin in the hair follicle and is not an immune-mediated phenomenon.[168] Similar tumours are sporadically seen in untreated rats, mice and hamsters.[159,164,169]

Other neoplasms of the skin showing aspects of hair follicular differentiation have been reported as *trichoepitheliomas* or *pilomatrixomas* in mice, rats and hamsters.[142,163,164,170] However, it should be noted that not all of these lesions fall into distinct categories and 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 tumours in humans but much less common in laboratory rodents, including the hairless mouse strains exposed to ultraviolet radiation.[145] Typical basal cell tumours 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 tumour cells.

Squamous carcinomas are the most prevalent form of skin tumour in rodent skin treated with carcinogen or irradiated with ultraviolet light. They are invasive tumours 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 tumours show differentiation towards hair follicles, although such neoplasms are usually all classified as squamous carcinomas.

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. Squamous carcinoma cells may be particularly

pleomorphic and show individual keratinization or spindle cell differentiation resembling mesenchymal cells. It has been shown that some of the spindle cell tumours that occur in the skin of transgenic Tg.AC mice are poorly differentiated carcinomas because they contain cytokeratins and desmosomes.[171] These mice which carry the *v-Ha-ras* transgene rapidly develop papillomas and carcinomas following topical treatment with promoters and carcinogens.

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.[139,140,142] Hairless mice are particularly predisposed to develop carcinomas showing epidermal appendage differentiation in response to ultraviolet light.[145]

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.[172] *Solar keratosis* is characterized by hyperkeratosis, parakeratosis, acanthosis and collagenous thickening of the upper dermis. Solar elastosis may also be observed (see above).

Neoplasms of the melanogenic system

The epidemiologic evidence implicates sun exposure as a major risk factor in melanoma development in humans.[173] DNA damage caused by ultraviolet radiation has a central role in the pathogenesis of these tumours. 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.[174]

A slight excess of malignant melanomas has been reported in patients on immunosuppressive therapy.[175] An increased number of benign naevi has also been reported in children on cancer chemotherapy.[176] 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.[177]

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.[159,164,178–182] Neoplasms of melanin-producing cells are also widespread among certain domestic animals, particularly in heavily pigmented species.[162]

Melanomas can also be induced in pigmented rodents such as the hamster and C57BL/6 mice by the cutaneous application of carcinogens.[142,183]

Melanomas have also been induced in animals by exposure to ultraviolet radiation.[184,185]

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 usually described as naevi in man.[161]

Naevi (benign melanoma)

As in humans, junctional, compound and intradermal naevi are recognized in animals.[161] A *junctional naevus* is one in which clusters or nests of rounded or polygonal melanocytic cells are present at the dermal–epidermal junction. In the *intradermal naevus*, nests or bundles of well-differentiated, rounded melanocytes are located exclusively in the dermis. The so-called *compound naevus* combines features of both junctional and intradermal types.

Intradermal naevi resembling the so-called blue naevus described in humans are also found in laboratory animals. This naevus is found in the dermis and 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.[186]

Malignant melanoma

These neoplasms show broadly similar histological patterns to benign naevi 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 both spread along the epidermis or 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.[187] 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.[178] 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.[159] Ward found only two malignant melanomas in 5,065 pigmented B6C3F1 mice.[164]

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 naevi similar to human blue naevi, through to premalignant cellular blue naevi.[183]

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 tumours

and normal tissues also contain S100. However, studies with the electron microscope have shown intracytoplasmic premelanosome, single membrane bound organelles containing membranous filaments.[188,189]

Subcutaneous (soft tissue or mesenchymal) neoplasms

The histopathological diagnosis of soft tissue neoplasms remains one of the more difficult issues in tumour pathology. However, these neoplasms are found relatively infrequently in routine rodent carcinogenicity bioassays and a simple classification is usually appropriate. Their characterization has much in common with the diagnosis of human soft tissue tumours where the actual classification used for clinical management is less complicated than the 'scientific' classification based on detailed patterns of tissue differentiation.[190]

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 agents not generally considered carcinogenic, may give rise to sarcomas around the injection sites after varying periods of time. Agents among the latter class include concentrated solutions of glucose and other sugars, sodium chloride, certain water-soluble food colourings and surfactants, carboxymethycellulose and macromolecular dextrans.[191–193] 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 tumour induction.[192]

Subcutaneous implantation of inert plastics and other materials of certain dimensions can likewise give rise to sarcomas around implantation sites in rodents, the so-called '*Oppenheimer effect*' or '*solid state carcinogenesis*' (Figure 2.4).[58,194,195] This phenomenon remains unexplained, for it does not fit easily into conventional concepts of tumour initiation, promotion and progression. More dramatic is the development of foreign soft tissue sarcomas around small glass and polypropylene covered microchips implanted into the subcutaneous tissues of heterozygous transgenic $p53^{+/-}$ mice within periods of as little as 15 weeks (Figure 2.5).[196] This small microchip uncommonly produces tumours in the usual laboratory strains of mice and rats. However, sarcomas have been induced by implanted microchips in Fischer 344 rats and various conventional mouse strains. Reported incidences in implanted Fischer rats is about 1% and in mice they range 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.[197–199] These differences suggest strain and species differences in sensitivity to this effect.

Chronic damage to tissues in other ways may also be associated with mesenchymal tumour development. One example is the development of spindle

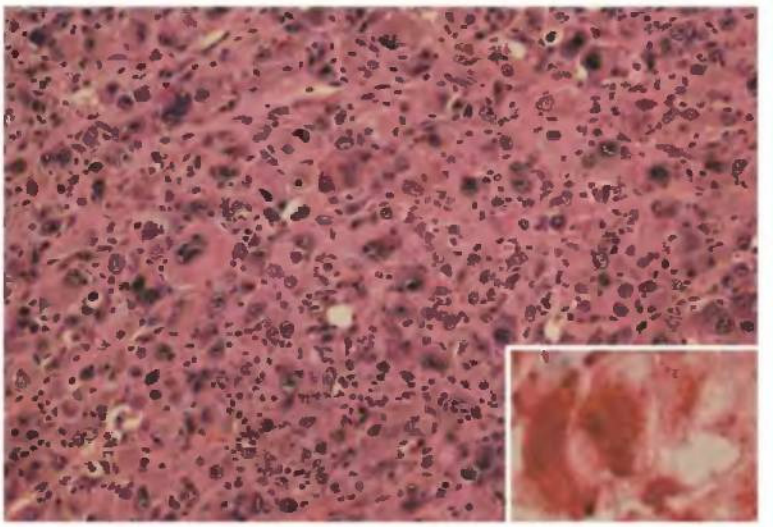

Figure 2.4 Pleomorphic sarcoma which developed in a rat implanted with a millipore filter 12 months previously. It shows the features of the so-called malignant fibrous histiocytoma reported in humans (H&E ×110). *Inset:* Histochemical reaction for acid phosphatase showing the intense lysosomal enzyme activity (frozen section azo dye reaction of Barka and Anderson ×300)

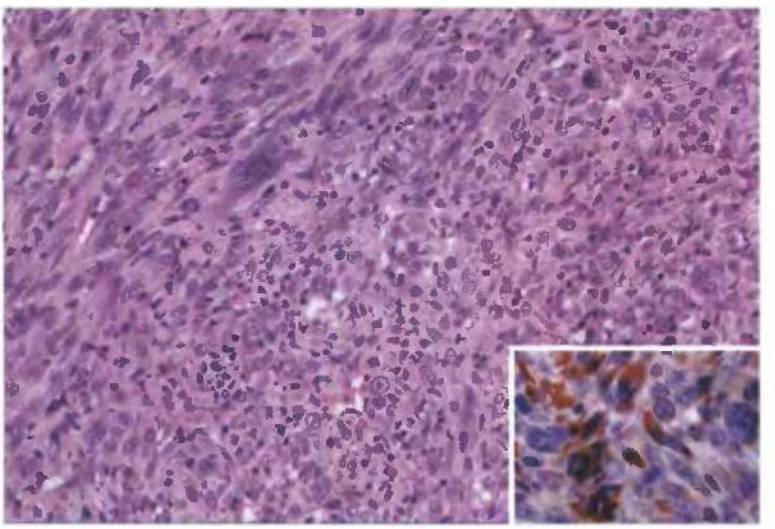

Figure 2.5 Sarcoma which developed around an electronic chip implanted in a $p53^{+/-}$ transgenic C57BL/6 mouse. It shows the typical pleomorphic appearance with spindle cells, giant cells and small round cells similar to sarcomas found in humans (H&E ×280). *Inset:* The same tumour stained for α smooth muscle actin (immunoperoxidase ×300)

cell neoplasms on the ear tips of rats treated for a long period with ergotamine, at the site of tissue damage produced by this agent.[200]

The development of sarcomas at injection sites is not generally considered to indicate potential cancer hazard to humans for agents administered by other routes.[201,202] The difficulties in interpretation are usually circumvented by avoiding administration by the subcutaneous route for long term toxicity studies. However, the increasing need 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 plastic biomaterials may necessitate consideration of these local effects at injection or implantation sites. Moreover, 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.

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.[191] 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.[195] As wound healing is modulated by numerous cytokines, including transforming growth factors, fibroblast and platelet-derived growth factors, tumour necrosis factor α, interleukin 1, colony stimulating factor 1 and vascular endothelial growth factor,[14] it is probable that some of these factors have an important role in the proliferative responses to implanted materials.

Although no clear relationship between the type of tissue response and chemical structure of the injected or implanted material can be 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.[192,202] In the case of subcutaneous implants, size, shape and form appear to be the most critical elements in sarcoma development in animal models.[58] This latter situation parallels the tumorigenicity of fibrous mineral particles in rodents that appears to be largely dependent on dimensions and durability of fibres rather than their precise chemical structure.[203] A recent study by Kirkpatrick and colleagues showed that cellular atypical foci develop close to the implants prior to the appearance of frank neoplasms.[204] These authors showed that cells in these foci were detectable by their high labelling index with antibody to proliferating cell nuclear antigen (PCNA).

In contrast to the tissue responses around inert plastics and the various non-carcinogenic agents, it has been shown that the early tissue response