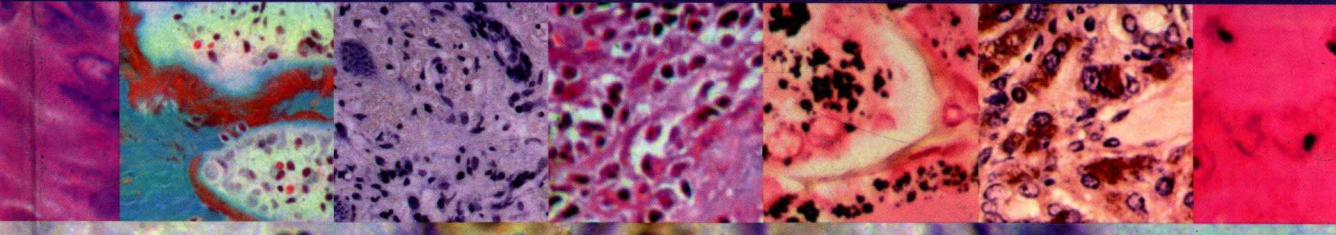
Fourth Edition

Histopathology of Preclinical Toxicity Studies

Interpretation and Relevance in Drug Safety Evaluation



Peter Greaves

HISTOPATHOLOGY OF PRECLINICAL TOXICITY STUDIES

INTERPRETATION AND RELEVANCE IN DRUG SAFETY STUDIES

FOURTH EDITION

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Contents

CHAPTER

1

Introduction

Pathology and the safety assessment of new medicines

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

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

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

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

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

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

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

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

Veterinary medicines

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

Toxicological screening

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

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

Carcinogenesis assessment

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

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

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

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

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

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

Comparative pathology

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

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

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

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

Techniques in pathology

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

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

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

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

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

Reporting of pathology findings

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

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

The following chapters

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

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

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CHAPTER

2

Integumentary System

SKIN AND SUBCUTANEOUS TISSUE

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

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

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

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