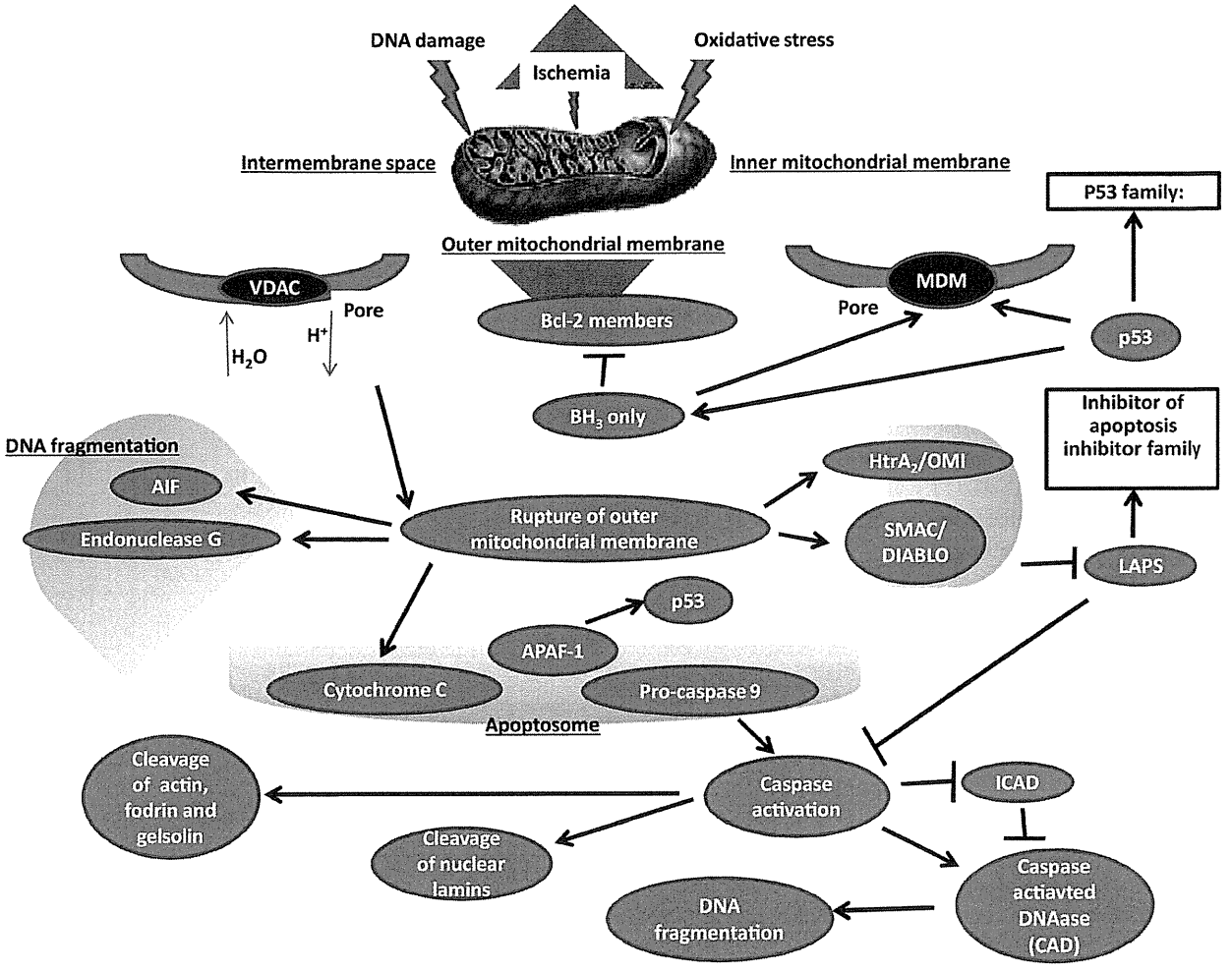

Figure 2. Intrinsic pathway.

inhibitory proteins. Once activated, NF-κB exhibits both anti-apoptotic and pro-apoptotic functions. Physiologically, NF-κB induces resistance to apoptosis through activation of inhibitor of apoptosis (IAP) and X-linked IAP. In addition, NF-κB activation has been shown to inhibit p53-dependent apoptosis following expression of the oncogene AP12/MALT1 [66]. This NF-κB directed survival response is associated with increased expression of anti-apoptotic proteins. Thus, it is not surprising that NF-κB expression is dysregulated in various disease states including chronic inflammation and cancer. In contrast, some stimuli that lead to activation of NF-κB may induce apoptosis, probably via the activation of pro-apoptotic proteins such as c-myc, p53 and caspase-1 [67].

The PI3K signaling pathway is also crucial for many aspects of cell growth, survival and tissue neo-vascularization and is frequently up-regulated in many cancers [68]. The PI3Ks are a family of related enzymes that are capable of phosphorylating the 3 position hydroxyl group of the inositol ring of PI(4,5)P2 to generate PI(3,4,5)P3 [69]. Upon activation of the PI3K pathway by many growth factors such as epidermal growth factor (EGF), PI(3,4,5) P3 is produced on the inner side of the plasma membrane and binds to Akt. Akt inactivates pro-apoptotic factors such as Bad, which controls the release of cytochrome c [70,71], procaspase-9 and Forkhead transcription factors (such as FOXO). Akt also activates anti-apoptotic genes including the cyclic-AMP response element-binding protein and IKB kinase leading to NF-κB nuclear localization and the subsequent transcription of pro-survival genes such as Bcl-xL, caspase inhibitors and c-Myb [72,73]. Over-expression of Akt has anti-apoptotic effects in various cell types resulting in resistance to cell death [74].

4. CANCER CHEMOPREVENTION AND APOPTOSIS

Cancer is a pathologic condition where the normal mechanisms of cell cycle regulation are dysfunctional either due to excessive cell proliferation, insufficient apoptosis

or both [75-77]. Suppression/inhibition of apoptosis during carcinogenesis is known to play a role in the development and progression of cancers [77-79]. At present, it is accepted that cell populations are tightly regulated by their rates of proliferation, differentiation and death. When the homeostatic balance is disrupted in such a way that clonal outgrowth of mutated cell populations occurs, the development of a tumor will followed [77-79].

In simple terms, one can define carcinogenesis as a multistage process where a normal cell becomes transformed into one with a malignant phenotype. Cells become initiated by the acquisition of an activating mutation in an oncogene or an inactivating mutation in a tumor suppressor gene (initiation). Several additional factors confer these cells a growth advantage (promotion), which allow the cell to survive while accumulating abnormal characteristics and ultimately progressing to a metastatic tumor (progression) [80]. Carcinogenesis is a complex process driven by tight interactions between oncogene activation, tumor suppressor inactivation and cell death machinery. Early in transformation, activated oncogenes that drive the cell to uncontrolled proliferation simultaneously trigger apoptosis, probably as a safety mechanism to remove cells carrying oncogenic mutations [81]. Later in tumorigenesis, the supply of nutrients and oxygen becomes limited, with the tumor cells undergoing hypoxia-induced apoptosis [82]. In order to survive, tumor cells acquire apoptotic-inhibiting mutations (reduced apoptosis) [82]. Failures in normal apoptotic pathways contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of mutations, promoting resistance to immune-based destruction, overriding cell-cycle checkpoints (that would normally induce apoptosis), facilitating growth factor/hormone-independent cell survival, supporting anchorage-independent survival during metastasis, reducing dependence of oxygen and nutrients, and conferring resistance to cytotoxic anticancer drugs and radiation [77]. Thus, inhibition of apoptosis can lead to tumor development.

In animal models, most chemical initiators are unable to initiate tumor growth unless a tumor promoter is subsequently applied. Many tumor promoters inhibit apoptosis *in vitro* [83]. Activation of apoptosis is thus being considered to be one of the most promising therapeutic approaches in cancer therapy [63,77,84]. Tumor cells can acquire resistance to apoptosis, for instance, by overexpressing anti-apoptotic proteins such as Bcl-2 or down-regulatinf/mutating pro-apoptotic proteins such as Bax, the expression of both being regulated by the p53 tumor suppressor gene [85,86]. p53 is a transcription factor essential for the prevention of cancer formation, which can be damaged by radiation, several chemicals and viruses such as human papillomavirus (HPV). The p53 pathway is ubiquitously lost in human cancer either by p53 gene mutation or by loss of cell signaling upstream and downstream of p53 in cancers that express the WT p53 gene [87]. Therefore, despite the enthusiasm towards apoptosis based-drugs, possible difficulties are also being anticipated such as selection of apoptosis-resistant tumor cells and systemic toxicity [84].

Several epidemiological studies, later evaluated by meta-analysis, have identified associations between certain dietary factors and cancer that either increase or decrease cancer risk [15,88,89]. It is currently accepted that diet can affect the overall process of carcinogenesis by different mechanisms: its constituents may contain cancer-causing substances as well as many cancer preventive agents. These dietary agents can retard or prevent the process of carcinogenesis by multiple mechanisms, namely 1) enhanced detoxification of the carcinogenic intermediates through induction of phase 2 drug metabolizing enzymes, 2) reduced carcinogenic activation due to suppression of cytochrome P450-dependent monooxygenases, 3) perturbations in cell cycle progression, 4) selective promotion of apoptosis in cancerous or precancerous cells, and 5) inhibition of angiogenesis and metastasis formation [16]. Since apoptosis provides a physiologic mechanism for eliminating abnormal cells, dietary factors affecting apoptosis can have an important effect on carcinogenesis. Conceivably, dietary factors that activate apoptosis in pre-cancerous cells offer a cancer preventive mechanism. In fact, most initiated cells are destroyed by apoptosis before they become malignant and develop into a tumor [79]. Increased understanding in the field of cancer has led to the conviction that most human malignancies should be fought on multiple fronts: in addition to cancer therapy, cancer prevention has become an important means of controlling cancer [8]. Common prevention strategies include avoiding exposure to known cancer-causing agents, enhancement of host-defense mechanisms against cancer, life style modifications and chemoprevention [8].

The term chemoprevention refers to the use of agents to slow the progression of, reverse or inhibit carcinogenesis, and was first introduced by Sporn and co-workers in the mid-1970's [90]. Animal studies, clinical trials and *in vitro* studies have examined the anticancer activity of numerous putative chemopreventive agents. These studies strongly suggest that the anti-cancer activities of many of these compounds involve the induction of apoptosis, and support the notion that apoptosis is a novel target for cancer chemoprevention [8,10]. Moreover, the pro-apoptotic properties of a variety of chemopreventive agents, like those of many conventional and experimental cancer chemotherapeutic agents, appear to be related to mitochondrial alterations in tumor cells [10,91]. In fact, several classes of chemopreventive agents contain members that trigger mitochondrial disruption and/or mitochondrial-

mediated apoptosis (intrinsic pathway) in tumor cells *in vitro*, although other agents may induce apoptosis via the death receptor pathway [9].

Chemotherapy aims to kill cancer cells, in the hope of preventing further cancer progression. Chemoprevention, on the other hand, involves administering non-toxic agents to individuals who may be at an increased risk for cancer. Moreover, surgical and traditional therapeutic approaches (chemotherapy and radiation) are, at present, unable to control most cancer types. Thus, the development of new chemopreventive strategies is required [10,11,92,93]. Chemopreventive compounds can be classified into two major groups: i) blocking agents, which prevent carcinogens from reaching or reacting with critical target sites, and ii) suppressing agents, which stop the evolution of the pre-neoplastic process. Given that the initiation and progression phases are relatively transient and irreversible events, it seems logical that chemopreventive agents should intervene at the prodromal promotion phase. Three decades of research suggest that chemoprevention is a promising strategy to reduce the incidence of cancer, both in well-defined high-risk groups and in the general population [10-12,92,93].

Of great importance, aberrant NF-κB regulation and Akt activation has been observed in many cancers. To prevent the development and progression of cancers, the strategy should target the cell signaling pathways that are deregulated in malignant tumors. Aberrant regulation of NF-κB and the signaling pathways that control its activity are involved in cancer development and progression, as well as in drug resistance, especially during chemotherapy and radiotherapy [94]. Blocking NF-κB can cause tumor cells to cease proliferation or become more sensitive to the action of antitumor agents[95]. Changes in Akt activator expression observed in human precancerous tissues that might be targeted for chemoprevention[96]. Inhibition of Akt signaling has been associated with the biological actions of numerous chemopreventive compounds. Thus far, several chemopreventive agents including, green tea polyphenols, curcumin, and quercetin have shown their various activities in the inhibition of carcinogenesis through the regulation of major cell signaling pathways such as Akt and NF-κB. Therefore, those are the subject of intense study. Agents capable of suppressing Akt and/or NF-κB activation have therapeutic promise and potential to inhibit carcinogenesis. We currently review dietary cancer chemopreventive compounds include EGCG, curcumin, and quercetin, capable of functioning in this capacity.

Representative dietary cancer chemopreventive compounds include EGCG, curcumin, and quercetin.

4.1. EGCG

Cancer prevention by green tea and its constituents been studied in different animal models of carcinogenesis [97]. The major catechins (a group of polyphenols) in green tea are EGCG, (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallete (ECG) and (-)-epicatechin. EGCG, the most abundant and most studied catechin, exhibits significant growth inhibitory effects in cancer cells. More importantly, after treatment of a primary cell line such as normal epithelial cells with EGCG, there is no observable toxicity at doses that are used for cancer inhibition studies [98, 99]. A collection of reviews have commented on the possible mechanistic effects of EGCG in multiple cell lines and have noted similarities in regard to growth inhibition and cell cycle arrest [100-102].

EGCG reportedly affects the transcription factors p53 and NF-κB leading to a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis [103]. The induction of apoptosis by other green tea catechins has been evaluated in a dose dependent manner (*i.e.* ECG > EGCG > EGC > EC) [104].

EGCG treatment may lead to a significant dose- and time-dependent inhibition of activation and translocation of NF-κB to the nucleus by suppressing the degradation of IKBα in the cytoplasm [105,106]. EGCG may also inhibit the ATP- and IL-1β-induced activation of NF-κB [107].

EGCG has been found to inhibit PI3K/Akt activation, resulting in the modulation of Bcl-2 family proteins and leading to the enhanced apoptosis of bladder cancer cells [108]. EGCG has also been shown to inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis *in vitro* through suppression of VE-cadherin phosphorylation and inactivation of Akt, suggesting that EGCG has an inhibitory effect on the Akt signaling pathway [109, 110]. Further studies have also demonstrated that constitutive activation of Akt, EGFR and Stat3 was inhibited in both YCU-H891 head and neck squamous cell carcinoma and MDA-MB-231 breast carcinoma cell lines treated with EGCG [111].

A dose-dependent increase in p53 was observed after EGCG treatment of LNCaP cells, which carry WT p53, but not in DU145 cells carrying mutant p53 [112]. EGCG was also shown to stabilize p53 and cause up-regulation of its transcriptional activity, resulting in the activation of its downstream targets such as $p21^{WAF1}$ and Bax, and the induction of apoptosis [113]. In human liver cancer cells, a significant increase in the expression of p53 and $p21^{WAF1}$ protein that lead to cell cycle arrest was reported after EGCG treatment [114]. **Table 1** summarizes the action/mechanisms of three selected compounds that affect NF-κB and Akt-phosphatidylinositol 3-kinase (PI3K) activity *in vivo* system.

Administration of EGCG to $Apc^{min/+}$ mice, an animal model of human intestinal carcinogenesis, via their drinking fluid was found to significantly decrease small intestinal

tumor formation [115]. Shimizu *et. al.* have recently reported that EGCG prevents obesity-related colonic and liver tumorigenesis by inhibiting the phosphorylation of the insulin like growth factor-1 receptor (IGF-1R), extracellular signal-regulated kinase (ERK), Akt, glycogen synthase kinase-3beta (GSK-3β), signal transducers and activators of transcripton 3 (Stat3), and c-Jun NH_2-terminal kinase (JNK) proteins, and improving hyperinsulinemia in carcinogen-induced mouse model [116,117]. EGCG suppressed the growth of melanoma cells in nude mice with impaired angiogenesis by inhibiting PI3K/Akt signaling specifically in tumor-associated endothelial cells and peripheral blood-derived endothelial cells [118]. Continuous feeding with EGCG to mice prior to and during establishment of bladder carcinoma xenografts *in vivo* revealed >50% reduction in mean tumor volume without detactable toxicity [119].

4.2. Curcumin

It is widely accepted that curcumin, a yellow pigment found in the rhizome of the spice turmeric, has potent cancer chemopreventive activity in various animal carcinogenesis models [120]. Curcumin is known to be a strong inhibitor of NF-κB. Curcumin has been shown to inhibit IKK, suppress both constitutive and inducible NF-κB activation, and potentiate tumor necrosis factor (TNF)-induced apoptosis [121]. Recent studies have shown that curcumin suppresses the constitutive activation of NF-κB [122] and sensitizes human colorectal cancer xenografts in nude mice to gamma-radiation by targeting NF-κB regulated gene products [123]. Treatment with a liposomal formulation of curcumin resulted in a dose-dependent growth suppression of cancer cells and decreased activation of NF-κB [124]. The findings that expression of NF-κB target genes, including cyclin D1, cyclooxygenase (COX)-2, matrix metalloproteinase-9, Bcl-2, Bcl-xL, Mcl-1L and Mcl-1S, was reduced by the treatment with a liposomal formulation of curcumin indicate that curcumin acts the NF-κB pathway which is involved in carcinogenesis. Al-Hujaily *et. al.* have demonstrated that a novel curcumin analogue, PAC, significantly reduced tumour size, and triggered apoptosis in breast cancer tumor xenografts by inhibiting expression of survivin, NF-κB and its downstream effectors, and strongly up-regulated p21 (WAF1) [125]. Moreover, clinical trials have shown that curcumin down-regulates the expression of NF-κB and COX-2 in peripheral blood mononuclear cells from patients with pancreatic cancer [126]. These results clearly indicate that curcumin inhibits tumor growth by affecting the NF-κB signaling *in vitro* and *in vivo*.

Curcumin also exhibits an inhibitory effect on Akt signaling. Recent studies have shown that curcumin dose- and time-dependently inhibits the phosphorylation of Akt and mammarian trget of rapamycin (mTOR), and their downstream targets in prostate cancer cells [127]. Inhibition of the Akt/mTOR pathway by curcumin results in suppression of the growth of SCC40 xenografts, and curcumin at 15 mg significantly increases in survival in the 4-nitroquinoline 1-oxide--induced head and neck squamous cell carcinoma survival study [128].

Curcumin has also been shown to inhibit the proliferation of cisplatin-resistant ovarian cancer cells via the inhibition of Akt activation [129]. It has also been reported that an analog of curcumin, 4-hydroxy-3-methoxybenzoic acid methyl ester (HMBME), targets the Akt signaling pathway, inhibits the proliferation of cancer cells and induces apoptosis [130]. Likewise, HMBME was shown to decrease the level of phosphorylated Akt, inhibit Akt kinase activity, and reduce the DNA-binding activity of NF-κB [130]. Several reports also suggest that curcumin has molecular targets within the Akt signaling pathways and that the inhibition of Akt activity may facilitate inhibition of proliferation and induction of apoptosis in cancer cells [131,132]. A curcumin derivative, diphenyl difluoroketone, significantly inhibited the colon cancer xenograft Akt and ERK phospholyration in mice [133]. In other study, solubilized curcumin effectively blocked brain tumor formation in the mice that had already received an intracerebral bolus of mouse melanoma cells (B16F10) by suppressing p-Akt, Cyclin D1, p-NF-κB, Bcl-xL and VEGF [134].

4.3. Quercetin

Quercetin, a powerful anti-oxidant, is consumed by humans as part of their diet [135]. Although the quercetin content of foods has not been systematically analyzed, it is found in many fruits, vegetables and beverages. The chemopreventive effects of quercetin against DNA damage and precancerous changes in cells were recently demonstrated both *in vitro* and *in vivo*. Quercetin was found to arrest the progression of cervical neoplasia in Swiss albino mice [136].

Quercetin may offer a defense against the detrimental effects of carcinogenic chemicals and can induce apoptosis via the mitochondrial pathway [137]. Although the anti-carcinogenic mechanisms of quercetin are not well known, quercetin specifically inhibits p21-Ras expression in human colon cancer cell lines and in primary colorectal cancers [138]. Reports suggest that quercetin has DNA-damaging and pro-oxidant property in cells [139]. One proposed mechanism involves the inhibition of Akt/protein kinase B (PKB) phosphorylation, an upstream kinase of the pro-survival protein kinase cascade involveing PI3K. Significant down-regulation of Bcl-2 and Bcl-xL along with Cu-Zn SOD, which could lead to an increase in ROS, has been reported after quercetin treatment in certain human cancer cell lines [43,140].

Survivin, which binds directly to and inactivates caspases was inhibited by quercetin, resulting in the activation of caspases [43]. One promising therapeutical approach for the induction of apoptosis in cancer cells is using TNF-related apoptosis-including ligand (TRAIL) [141]. Quercetin has been shown to potentially arrest human prostate cancer cells and mediate activation of caspase and poly(ADP-ribose)polymerase (PARP) cleavage [142,143]. A combined treatment of TRAIL and quercetin was found to enhance TRAIL-induced cytotoxicity by activating caspases and inhibiting phosphorylation of Akt [144].

A principal approach for treatment of tumors is based on the fact that cancer cells are resistant to CD95-mediated apoptosis. Alterations in the CD95 system result in the escape of tumor cells from this defense system. Sensitivity to CD95 can be restored by treating the cells with quercetin, thus making the cancer cells susceptible to apoptosis [145]. One study has demonstrated the inhibitory effects of quercetin on H_2O_2-induced apoptosis via mediation of the AP-1-mediated apoptotic pathway. The mechanistic action of quercetin as an anti-apoptotic compound is attributed to its ability to inhibit MAPK pathways and reduce expression of genes participating in the JNK-c-JUN/AP-1 and ERK-cFOS/AP-1 pathways [146].

In an azoxymethane (AOM)-induced rat colon cancer model, dietary administration wirh quercetin and curcumin decreased the number of aberrant crypt foci (ACF), putataive precursor lesions for colonic adenocarcinoma, by 4- and 2-fold, respectively, compared with the controls [137]. Western blot analyses of caspase-9, Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) proteins from colon scrapings suggest that quercetin and curcumin induce apoptosis via the mitochondrial pathway. Sun *et al.* reported that quercetin significantly prevented *in vivo* growth of human salivary adenoid cystic carcinoma xenografts in nude mice, accompanied by induction of tumor cell apoptosis, suppression of NF-κB nuclear translocation, as well as down-regulation of Akt and IKB kinase-α activation. Thus, quercetin would be a promising chemotherapeutic agent through its function of down-regulating the PI3K/Akt/IKK-alpha/NF-κB signaling pathway [147].

5. COMBINATION THERAPY

Some naturally occurring chemopreventive compounds are known to act in synergy with other chemopreventive or anti-cancer agnets, as listed in **Table 2**. The anti-neoplastic agents have dose-limiting toxicity and drug resistance, thus limiting their clinical application. Development of novel strategies that overcome radio- and chemo-resistance and sensitize cancer cells to anti-neoplastic agent can enhance the therapeutic effect of these drugs. Combination treatment with EGCG and tamoxifen was synergistically cytotoxic and enhanced apoptosis in MDA-MB-231 human breast cancer cells and decreased tumor growth in a MCF-7 cell xenograft model [148,149]. The combined treatment with EGCG and curcumin also resulted in synergistic growth inhibition of MDA-MB-231 [150]. Curcumin and gemsitabin treatment decreased pancreatic tumor volume *in vivo* model [151,152]. Furthermore, combination of quercetin with sulforaphane, an isothiocyanate enriched in broccoli, exerted synergistic effects by improving apoptosis resistance [153]. Liposomal forms of curcumin plus resveratrol significantly decreased prostatic adeno-carcinoma in prostate-specific PTEN-knockout mice by effectively inhibiting cell growth and induceing apoptosis [154].

Bcl-2 family proteins are regulators of chemoresistance and radioresistance in cancer. *In vivo* treatment with quercetin and trans-3,5-dimethoxy-4'-hydroxystilbene (t-PTER) altered expression of molecules involved in regulating cancer cell resistance to drugs and radiations [155]. Combined administration with t-PTER+ quercetin, FOLFOX6 (oxaliplatin, leukovorin, and 5-fluorouracil, a first-line chemotherapy regimen), and radiotherapy eliminates colorectal cancer cells growing *in vivo* leading to long-term survival. Gene expression analysis of a Bcl-2 family of genes revealed that down-regulation of bcl-2 expression via inhibition of NF-κB activation. Curcumin also potentiates the antitumor effects of radiation therapy in colorectal cancer by suppressing NF-κB and NF-κB–regulated gene products, leading to inhibition of proliferation and angiogenesis [123]

In mouse cervical multi-stage squamous cell carcinoma model using 3-methylcholanthrene and a xenograft model of human cervical cancer in mice, the combined treatment with curcumin and paclitaxel induced a synergestic reduction in the tumor incidence as well as tumor volume compared with the individual treatment of paclitaxel or curcumin[156],suggesting that a suboptimal concentration of curcumin augments the anti-tumor action of paclitaxel by downregulating the activation and downstream signaling of anti-apoptotic factors and survival signals such as NF-κB, Akt and MAPKs that have significant roles in proliferation, survival, angiogenesis and metastasis. By inhibition of NF-κB activity, curcumin augments the anti-tumor action of cisplatin enhancing growth suppression *in vivo* [157].

Recently, preclinical investigation revealed that combination therapy with curcumin and dasatinib to be highly effective causing an over 95% regression of intestinal adenomas in $Apc^{min/+}$ mice, which could be attributed to decrease proliferation and increased apoptosis [158].

6. SUMMARY AND PERSPECTIVES

The pathogenesis of many chronic diseases, including cancer, has been associated with aberrantly regulated apoptosis [14,75,77,78,159]. The synergistic combination

of an undesirable proliferative stimulus and an associated defect in the apoptotic pathway seems universal in cancer [76-80]. Epidemiological studies indicate that dietary habits contribute to, at least, one third of all human cancers [160], and suggest that certain dietary components can exacerbate or interfere with carcinogenesis. Apoptosis is likely to be a crucial mechanism in the chemopreventive properties associated with such dietary factors [14].

In addition to the conventional therapeutic agents, numerous dietary components and micronutrients are emerging, which possess considerable potential for hindering *in vivo* deleterious oxidative processes and inducing apoptosis of cancerous or pre-cancerous cells [160,161], and are therefore being considered as promising chemopreventive agents. A range of dietary compounds can modulate apoptosis and those with pro-apoptotic properties exhibit beneficial effects in animal and *in vitro* studies by eliminating cancerous cells [9,14,92]. Moreover, some dietary compounds have also shown beneficial effects in clinical trials [93].

A balance of cell proliferation and apoptosis normally maintains cellular homeostasis. However, apoptosis is a very complex process with numerous specific targets within each arm of the apoptotic pathways. Nevertheless, it is very encouraging that single bioactive dietary agents can directly and indirectly influence many of the targets within the apoptotic pathway. In additon, many of these dietary agents appear to exhibit some degree of specificity for neoplastic cells. Furthermore, the protective effects of single agents can be potentiated and/or synergized by other dietary factors suggesting the possibility of combinational approaches for chemoprevention. While dietary interventions seem encouraging for devising new chemopreventive strategies, there are several issues remaining that need to be fully understood. The dose of each agent, duration of exposure, relative bioavailability of each dietary compound and potentially adverse side effects and/or interactions should be considered.

Further research is required to identify the phytochemical-specific molecular mechanisms of the huge number of already recognized bioactive dietary chemopreventive agents. The potential benefits of cancer chemoprevention appear promising given the data obtained from clinical trials and pre-clinical studies.

7. CONFLICT OF INTERST

The authors declare no conflict of interest.

8. ACKNOWLEDGEMENTS

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International Harmonization of Toxicologic Pathology Nomenclature: An Overview and Review of Basic Principles

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ABSTRACT

The International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice is a global project that is publishing criteria for both proliferative and nonproliferative changes in laboratory animals. This paper presents a set of general suggestions for terminology across systems. These suggestions include the use of diagnostic versus descriptive terms, modifiers, combination terms, and grading systems; and the use of thresholds, synonyms, and terminology for some processes that are common to several organ systems. The purpose of this paper is to help the reader understand some of the basic principles underlying the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice process.

Keywords: nomenclature; tumors; rodent pathology.

PRIOR HARMONIZATION PROJECTS

For many years, harmonization of nomenclature and diagnostic criteria in toxicologic pathology, especially for rats and mice, has been a goal of pathologists working in the profession. In the latter part of the twentieth century, several initiatives were undertaken by the Society of Toxicologic Pathology (STP) in the United States and by the Registry of Industrial Toxicology Animal-data (RITA) database group in Europe. Their efforts resulted in a number of internationally recognized publications, the Standardized System of Nomenclature and Diagnostic Criteria: Guides for Toxicologic Pathology (http://www.toxpath.org/ssndc.asp) and the World Health Organization/International Agency for Research on Cancer International Classification of Rodent Tumors.

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INTERNATIONAL HARMONIZATION OF NOMENCLATURE AND DIAGNOSTIC CRITERIA FOR LESIONS IN RATS AND MICE NOMENCLATURE PROJECT

Beginning in 2005, the STP and the European Society of Toxicologic Pathology, in conjunction with RITA, developed a collaborative process to review, update, and harmonize existing nomenclature documents and databases. In 2006, the British Society of Toxicologic Pathologists and the Japanese Society of Toxicologic Pathology joined the initiative, so that the project has become truly global. The goal of the project is to produce publications for each organ system that provide a standardized nomenclature and differential diagnosis for classifying microscopic lesions observed in laboratory rats and mice in toxicity and carcinogenicity studies. The project is referred to as the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND).

Briefly, the INHAND project is organized as follows. The Global Editorial and Steering Committee (GESC) oversees the activities of the project. The GESC is composed of toxicologic pathologists from all of the participating societies. In addition, there are several technical consultants for online and print publishing support (Figure 1).

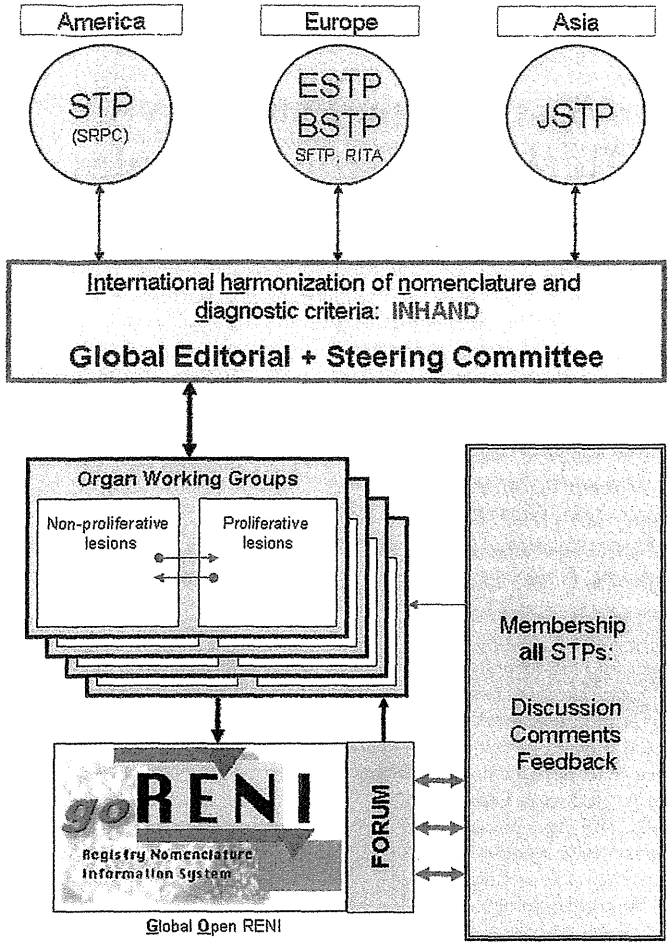

FIGURE 1.—Organization of INHAND.

The Organ Working Groups, composed of expert toxicologic pathologists from each of the participating societies, are the core of the project. Each group is responsible for developing preferred nomenclature and diagnostic criteria. Once the draft nomenclature has been developed, the GESC completes an initial review, followed by a period during which all members of participating societies are requested to review the proposed nomenclature. The Organ Working Group then finalizes the nomenclature based on comments from the GESC and general membership. An important feature of the INHAND project is the use of the global open Registry Nomenclature Information System (goRENI, http://wwwgoreni.org) as a Web-based platform to both review draft nomenclature and publish final nomenclature. Originally developed by RITA, goRENI provides access to members of all toxicologic pathology societies as well as government regulators.

In addition to goRENI, the completed nomenclature for each organ system will be published in one of the official journals of the participating societies: *Toxicologic Pathology* (STP and British Society of Toxicologic Pathologists), *Experimental and Toxicologic Pathology*, or the *Journal of Toxicologic Pathology*. The first systems to be published were the respiratory (Renne et al. 2009) and hepatobiliary (Thoolen et al. 2010) systems, and the remaining organ systems are targeted to be published by the end of 2013.

GENERAL CONCEPTS AND APPROACHES

The individual organ system publications provide information on specific diagnostic entities and differential diagnoses; however, certain key principles and processes are common across the various organ systems. The purpose of the following sections is to provide guidance on general approaches to recording microscopic lesions in toxicity studies. The following comments are complementary to the approaches and perspective provided by prior authors (Crissman et al. 2004; Dua and Jackson 1988; Haschek et al. 2010; Herbert et al. 2002; Shackelford et al. 2002; Wolf and Mann 2005).

Although the diagnosis of proliferative lesions is fairly straightforward (a mass either meets the criteria for diagnosis or it does not), nonproliferative lesions present a more formidable challenge. Different grading scales, whether a change should be graded, and the use of modifiers and thresholds all provide opportunities for significant variation between pathologists. In 2007, Greaves emphasized the importance of consistency in diagnosis, writing,

> "[I]t is salutary to remember that toxicologists and physicians in government regulatory agencies usually read the text relating to pathology findings with extreme care. In addition the tabulated summaries of pathology are often reviewed with equal attention. Unclear language, inappropriate, misleading or unexplained terminology, conclusions not justified by data, any discrepancy between text and tables may all raise unnecessary questions. Thus, clarity of the report and explanation of all findings are essential" (Greaves 2007).

Since the purpose of the INHAND project is to generate (so far as possible) standardized nomenclature for both proliferative and nonproliferative changes, the more important of these variables are discussed below.

Diagnostic versus Descriptive Terminology

Most pathologists are initially trained in interpretive diagnostic pathology, with the goal of rendering a definitive diagnosis through the use of morphologic, etiologic, and disease diagnosis. In rendering a final disease diagnosis, the pathologist is often incorporating a wide range of data including history, clinical signs, and laboratory data. In toxicologic pathology, the goal is to determine whether the test article produces changes through a comparison of treated animals with control animals. As such, it is important that microscopic observations be recorded in a consistent, objective manner that readily allows tabulation and comparison of group effects. In this setting, the use of descriptive, rather than diagnostic, terminology is preferred. In many cases, a disease diagnosis implies a particular pathogenesis or impact on organ function based on what is known about the spontaneous disease, which may be misleading in the experimental setting of

a toxicity study. For example, a pharmacologic agent may cause an increase in trabecular and cortical bone. If this observation was recorded as osteopetrosis, a specific disease state (osteopetrosis), mechanism (decreased osteoclast function), and etiology (genetic mutation) could be implied, when in fact the effect was caused by a pharmacologic process. Another issue frequently encountered in toxicologic pathology is determining how to document vacuolation in multiple tissues resulting from the process of phospholipidosis. In recording the observations rendered from the routine hematoxylin and eosin–stained section, the pathologist should document the morphologic diagnosis (cytoplasmic vacuolation). In the pathologist's report, additional data (ultrastructural or biochemical data) can be integrated to render the interpretation that the vacuolation is caused by the process of phospholipidosis. In some studies, pathologists are confronted with novel treatment-related changes that may require unique terminology; these changes need to be treated on a case-by-case basis. Consistently using descriptive, rather than diagnostic, terminology in tabulating anatomic pathology data will decrease confusion and misconceptions. Additional description and interpretation on the likely process can be most appropriately provided in the pathologist's report (narrative).

Modifiers

In addition to severity grades, to provide more information and clarity, several additional modifiers may be included in a diagnosis. It is important to remember that the next person to review a diagnosis may not have access to the slide and likely is not a pathologist. For these reasons, accuracy and consistency in diagnostic terminology are crucial. Modifiers that are commonly used include organ-specific topography, distribution, character of the change, and duration (Frame and Mann 2008).

Organ-specific topography varies with the anatomic complexity of the organ; topography may also vary because of the prevalence of treatment-related changes in the organ. For the liver, examples include acinar, portal, periportal, midzonal, centrilobular, hilar, ductal, periductal, pericanalicular, or subcapsular—all of which may be used to indicate the specific location within the organ where the change has occurred. For the kidney and thymus, it is often useful to differentiate cortical from medullary changes, whereas in the central nervous system, the anatomical location affected is often crucial to understanding the pathogenesis of the change.

Distribution modifiers are used to indicate the pattern of change. Commonly used distribution modifiers include focal, multifocal, and diffuse. Based on the formal definition, a focal lesion refers to one specific area, or focus, whereas multifocal refers to more than one focus (foci). However, some pathologists use focal for both focal and multifocal, referring to the nature of the lesion rather than its actual distribution and using grading to reflect the extent of the lesion (Thoolen et al. 2010). Diffuse lesions affect the majority of the section examined, whereas focally extensive may be used to describe a large lesion that affects a significant portion of the section, but which is still localized in nature.

A modifier for character of change becomes important when the general diagnostic term is quite broad. An example is necrosis. Character modifiers might include apoptotic, caseous, coagulative, single-cell, or bridging. The use of character modifiers is often predicated by the organ where the changes occur (i.e., single-cell or bridging necrosis may be important indicators in the liver).

Terms for duration of a change have been used in both natural disease processes and treatment-related changes. Commonly used terms for duration include acute, subacute, chronic, and chronic-active, which is used to identify a lesion of chronic duration that also has ongoing acute changes.

These terms are generally associated with a particular type of inflammatory cell: acute (neutrophilic), subacute (neutrophilic mixed with mononuclear cells, lymphocytes), chronic (lymphocytes, plasma cells, mononuclear cells, or macrophages), and chronic-active (both mononuclear cells and neutrophils). For routine toxicology studies, the recommendation is to forego chronicity modifiers and rely on cell type or process (fibrosis, hyperplasia, etc.), especially since toxicology studies last a set number of days and the morphology represents a single point in time. For natural disease, generally there is more known about the time course of effects, so acute, chronic-active, and so on have more meaning.

Not all of these modifier types are used by every pathologist for all situations. However, having the terms in our armamentarium and using them consistently will help immensely in producing clear tables and reports. A model of how a pathologist might construct a descriptive diagnosis using modifiers is presented in Figure 2.

Combination Terms

For some common findings that can involve a combination of morphologic responses, compound-term diagnoses have been created to reduce the number of terms that need to be selected in pathology data entry systems. For example, degeneration may be present with concurrent evidence of regeneration in various tissues, and therefore the two processes are often combined into a single morphologic diagnosis of "degeneration/regeneration" at the discretion of the pathologist. Another approach has been to use terms such as "nephropathy, cardiomyopathy" for organ-specific changes summarizing a constellation of histologic features. Combination terms may also be appropriate for longer-term studies, including carcinogenicity studies, where there might be progression in toxicity (i.e., progressing from vacuolation to degeneration to necrosis to repair) over time. These compound terms are used with locators and modifiers in the usual way.

Grading Systems/Severity Scoring

Most toxicologic pathologists use a grading system to document lesion severity. In general, the grade of severity assigned to a diagnosis should be chosen to reflect a combination of the extent of the process (how many of its subordinate components are present), the distribution (focal to diffuse), and the actual

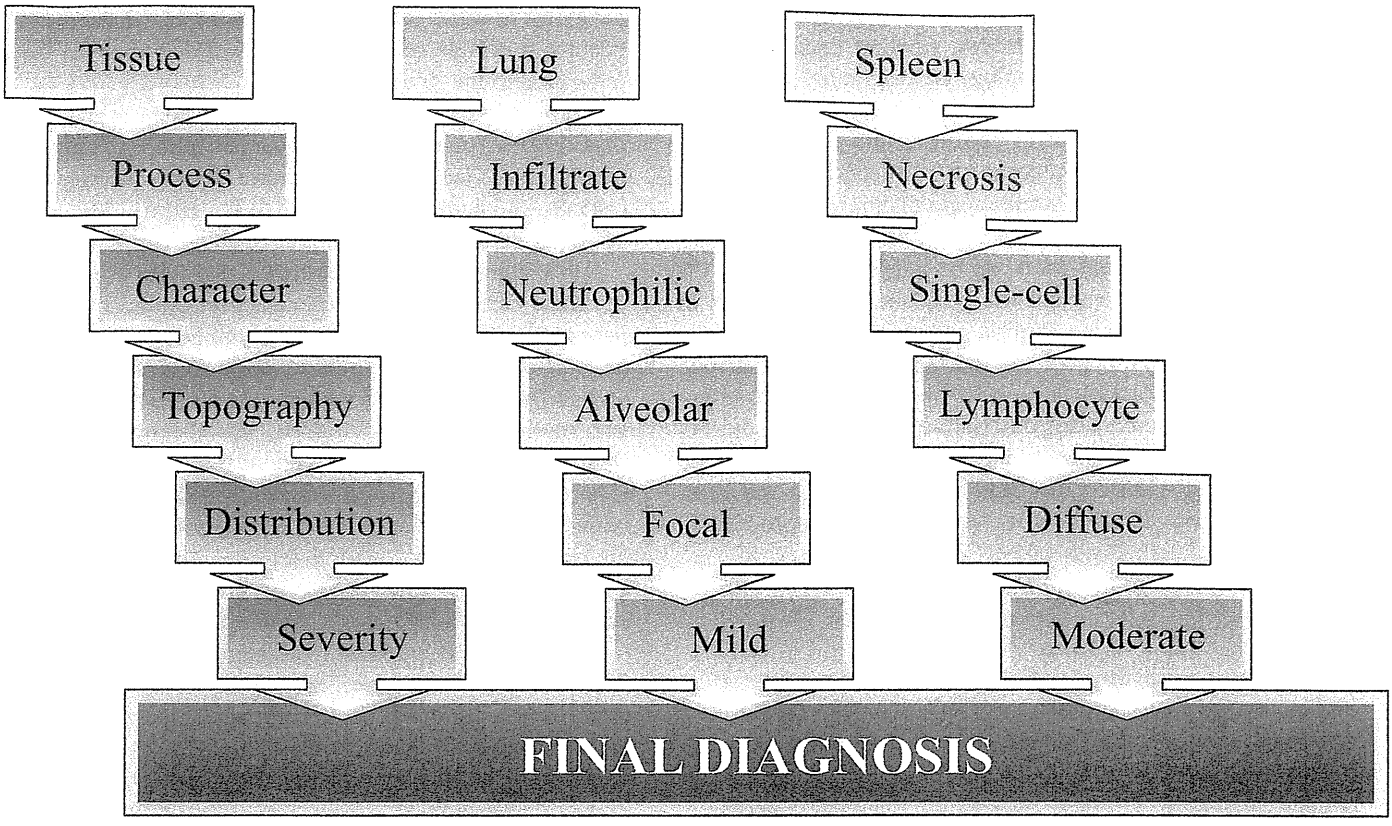

FIGURE 2.—Organization of a final diagnosis. Various modifiers and severity grades can be added to a basic key term or diagnosis to describe and categorize microscopic observations. In blue is an example of the fields possible in a diagnostic term, and examples are in red.

TABLE 1.—Illustration of a 4-point scoring system.

Numerical Score	Description	Definition
0	Within normal limits	Tissue considered to be normal, under the conditions of the study and considering the age, sex, and strain of the animal concerned. Alterations may be present, which, under other circumstances, would be considered deviations from normal.
1	Minimal	The amount of change present barely exceeds that which is considered to be within normal limits.
2	Slight	In general, the lesion is easily identified but of limited severity.
3	Moderate	The lesion is prominent, but there is significant potential for increased severity.
4	Severe	The degree of change is as complete as possible (occupies the majority of the organ).

degree of severity. Grading systems vary between laboratories and are often based on differences in the computerized pathology data capture systems. These systems may differ in how they incorporate distribution, stage, and extent of lesions. Within a given grading system, it is important to allow the individual study pathologist flexibility in grading to allow him or her to accurately demonstrate a dose response and characterize the toxicity of the test article. The problem of harmonization as it relates to lesion severity has been recognized and discussed in some detail (Hardisty and Eustis 1990; WHO 1978). Given this complexity and need for flexibility, a universal grading system would be difficult to achieve.

The most commonly used grading systems use either four or five distinct semiquantitative grades. An example of a four-point severity scale is shown in Table 1. The grades can either be expressed as numbers (where 0 indicates no change and 1–4 or 1–5 indicate increasing severity) or as words indicating the degree of severity, which correlate to the numerical score. Some systems have been suggested in which the percentage of the organ affected is used to assign a severity score (Shackelford et al. 2002), but this score can vary according to the organ involved. In addition, there may be a difference in the relative degree of severity assigned to a lesion depending on the length of the study (some changes may be graded as less severe in a chronic study than in a short-term study). Although the generation of ordinal data using a scoring system may allow statistical analysis for effects and trends (Gad and Rousseaux 2002), routine statistical analysis of severity grades is strongly

discouraged because the grading scale often is not linear, and the assigned number is only semiquantitative and is based on variable criteria. Determination of test article effects and dose response is most appropriately done by the study pathologist, rather than by statistical analysis.

Consistency in assigning severity grades within a study or between studies on the same compound is a key quality attribute of the pathology evaluation, and inconsistency or "drift" (the tendency for diagnostic criteria to vary over time) in assigning diagnostic grades within a study can either compromise the ability to detect a test article effect or lead to the appearance of a test article effect when none is actually present. In some cases, to convey a treatment-related effect or the dose-responsiveness of an effect, toxicologic pathologists may attempt to modify or "split" their criteria for severity scores in a study. For example, a pathologist may be able to reliably separate individual lesions into multiple groups, despite the fact that all the lesions represent variations of minimal. Although this process may be appropriate in some situations, it creates challenges in interstudy consistency and may create the impression that a change is more severe than the glossary-defined severity terms would suggest. If this approach is taken, the toxicologic pathologist should clearly specify the process and criteria in the methods section of the pathology report. During the peer review process, the reviewing pathologist should evaluate the consistency of grading within the study. As long as severity grades were consistently applied across a study, a one-point difference of opinion regarding severity is acceptable, given the semiquantitative nature of severity scoring.

Nongraded Changes

For some lesions, pathologists may not assign a severity grade, as no additional information would be gained. These changes are typically recorded as Present (P), rather than having a severity grade assigned. Examples include neoplasms, cyst(s), autolysis, and congenital anomalies. However, some pathology data entry systems require entry of grades for all lesions.

Thresholds

In addition to recording treatment-related alterations and notable spontaneous lesions, the pathologist must determine whether or not to document minor variations in normal tissue morphology. In many cases, these variations result from minor age-related changes, whereas in other cases, they represent normal anatomic variability within a population of animals. Thresholding refers to the practice of determining which variations in normal morphology will be recorded and which variations are below a threshold and will not be recorded. In a population of untreated animals, there are normal subtle variations in the morphology of tissues, so some degree of thresholding is needed to provide a meaningful compilation on microscopic pathology data. Factors the pathologist must consider in determining appropriate diagnostic thresholds include the morphology noted in the concurrent control animals and his or her understanding of normal morphology for the age, strain, and source of the test species. Great care must be taken in determining thresholds; indeed, some pathologists may employ limited, if any, thresholds. If a common spontaneous lesion is not recorded, the potential for a treatment-related effect on the incidence or severity of the change may be more difficult to detect. Alternatively, setting an appropriate threshold can aid in streamlining the number of diagnoses produced in a study, so that treatment-related changes are clear. Similar to other areas, the process of a pathology peer review will assist in ensuring appropriate diagnostic thresholds.

Synonyms

For all systems, the preferred term is listed at the start of each morphologic change. For many lesions, a list of synonyms (generally, previously used terms) follows the preferred term. In toxicologic pathology, the name of a lesion may evolve over time, although the morphology stays the same. Advancements in biology and understanding of how tissue alterations develop support the rationale for changing the terminology. In performing risk assessment, it is important that interpretation of data be based on current terminology and understanding of a particular lesion's biological significance. However, interpretation may also require review of historical data, which may have been collected and published years or decades earlier. The evaluation of older literature must take into account changes in terminology and the current knowledge and thinking on the significance of a lesion.

Pathology Processes Common to Organs

Vascular-Based Changes

Processes related to vascular changes, including congestion, hemorrhage, edema, and degeneration and/or inflammation of blood vessels, will be discussed in the cardiovascular system manuscript.

Infiltrate versus Inflammation

As discussed earlier, the use of descriptive, rather than diagnostic, terminology is preferred. In many cases, a diagnosis of inflammation, such as hepatitis, implies a particular pathogenesis, impact on organ function, or clinical syndrome. It is recommended that terms describing the type of cellular infiltrate are used rather than terms that describe the type of inflammation, particularly if the response is predominantly aggregation of cells without other features of inflammation. Thus it is preferred to describe a change in the kidney as consisting of infiltrate, neutrophilic rather than either acute inflammation or acute nephritis, since these latter terms are more appropriately used in clinical medicine. Most toxicologic pathologists would not use organ-specific disease descriptors (hepatitis, pneumonia, orchitis) but would build a diagnosis using the descriptors described above to accurately describe the change. The character of the descriptors may be more fully described in the pathology narrative.

On the other hand, if the constellation of changes that characterize active inflammation (including one or more of the

following: increased blood flow [congestion], microvascular exudation of plasma fluids and proteins [edema], and/or margination and emigration of leukocytes) are present, then a base term of inflammation followed by suitable descriptors may be appropriate. At the other end of the spectrum, more prolonged or chronic-active inflammation would be characterized by the collective features of ongoing tissue destruction, infiltration of a mixed inflammatory cell population, and variable extent of granulation tissue and/or fibrosis. As noted earlier in this discussion, it is best to avoid use of terms that may have a clinical or disease connotation such as "hepatitis."

Intracellular Accumulations

The recommendation is to start with a base term of "vacuolation," followed by modifiers as appropriate or more specific diagnosis following suitable special stains (Kumar et al. 2005; McGavin and Zachary, 2006).

Lipids: Lipids such as cholesterol, triglycerides, and phospholipids can accumulate in cells. This accumulation appears as clear vacuoles of various sizes and shapes and must be differentiated from accumulation of water or glycogen, which may sometimes produce vacuoles. Cholesterol accumulation can also appear as crystalline or cleft-like spaces. Since the process of fixation with paraffin embedding removes lipids, special stains such as oil-red-O are evaluated on frozen sections. More specific descriptions and differential diagnoses are discussed in organ systems.

Glycogen: Excessive amounts of glycogen can be seen with glucose or glycogen metabolism abnormalities. It can also be observed in the liver of animals not fasted prior to necropsy. Excess glycogen can appear as vacuoles or more indistinct clear spaces. It is best detected following fixation with alcohol and staining via periodic acid–Schiff reaction. More specific descriptions and differential diagnoses are discussed in organ systems.

Extracellular Accumulations

Hyaline change: Extracellular hyaline change or hyaline substance refers to an alteration in which homogeneous, glassy, eosinophilic material accumulates in tissue spaces, between cells or along basement membranes, or within the cytoplasm. Specific terminology and descriptions are addressed in organ systems, as appropriate.

Amyloid: The most common "hyaline" change is the accumulation of amyloid, a diverse group of glycoproteins. It is generally extracellular, compressing adjacent parenchymal cells, resulting in cell death from ischemia, and eventual atrophy. Congo red stains amyloid orange to orange-red. In polarized light, the presence of amyloid is shown by apple-green birefringence of the Congo red–stained sections.

Amyloid may also be demonstrated by immunohistochemistry. Specific organ responses and appearance will be reviewed in each organ system.

Cholesterol crystals: Cholesterol crystals are cleft-like deposits that may be present in areas of inflammation and represent a by-product of hemorrhage and necrosis; routine processing generally removes the deposit, leaving a clear angular space. Occasionally, these crystals can incite a granulomatous inflammatory response.

Mineralization

The recommendation is to start with a base term of mineralization (calcification) followed by modifiers, as appropriate. Tissue mineralization may be a result of several distinct processes.

Dystrophic calcification: Dystrophic calcification can occur in areas of necrosis; the calcified material may have a basophilic, granular, or clumped appearance. Progressive accumulation of layers can create a lamellate appearance (psammoma bodies). It can be intracellular or extracellular. Special stains to confirm include von Kossa and alizarin-red-S.

Metastatic calcification: Metastatic calcification occurs in normal tissue following syndromes that produce hypercalcemia such as hyperparathyroidism, destruction of bone secondary to neoplasms or genetic disease, renal failure, and vitamin D toxicosis. It can occur throughout the body, but gastrointestinal mucosa, kidneys, lungs, systemic arteries, and pulmonary veins are the most common sites. The morphologic appearance is similar to dystrophic calcification.

Osseous Metaplasia

Osseous metaplasia appears as distinct foci of immature woven bone or more dense lamellar bone. The pathogenesis of this change is not understood. It can occur throughout the body, but in rodents, it is most commonly found in the lung, brain, adrenal gland, heart, and eye.

Pigments

The recommendation is to use a base term of pigment, followed by appropriate modifiers (may be supported by special stains).

Exogenous pigment: Since most laboratory rodent species are bred and housed in a controlled environment with set humidity and filtered air circulation, exogenous pigments such as carbon are not common. Rodents that are identified with tattoos may have pigment within dermal macrophages and local lymph nodes.

Endogenous pigment: Melanin is a pigment normally present in the epidermis, retina, and iris of the pigmented rodent strains. Melanin may occur normally in the spleen and meninges of mice. Lipofuscin pigment is a fine, yellow-to-brown, granular pigment present in the cytoplasm (frequently perinuclear). It has

been noted as increasing with age and secondary to increased free radical injury and lipid peroxidation. Hemosiderin is formed from ferritin and is an intracellular, golden-yellow to golden-brown, and granular-to-globular pigment. Local or systemic excesses of iron result in hemosiderin accumulation. It can be easily visualized by Prussian blue stain.

Conclusions

Anatomic pathology is a descriptive and interpretive science and provides critically important information on the toxicity of environmental and biopharmaceutical agents. Toxicologic pathologists strive to provide clear and concise data and interpretations to toxicologists, physicians, regulatory reviewers, and others involved in risk assessment. The inherent variability of spontaneous "background" changes in our biological models, the complexity and variability of responses to the test article, and differences in diagnostic terminology, severity grading, and thresholds present challenges in consistently delivering clear interpretations. Application of the principles described in this paper and the consistent use of the harmonized nomenclature recommended in the INHAND publications will continue to increase the clarity and quality of anatomic pathology data in toxicity studies.

There are a number of possible grades and terms that may be used to describe nonproliferative lesions encountered in a toxicology study. Although there will always be some difference between the criteria used by individual pathologists, it is hoped that the descriptions given in this paper will help achieve some degree of international agreement and consistency as to the use of terminology to produce clear, concise data. It is hoped that the international character and review of the INHAND documents ensure that use by pathologists and national regulatory agencies will aid in the safety assessment of drugs, biologics, and chemicals.

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