REVIEW

Hemangiosarcoma in Rodents: Mode-of-Action Evaluation and Human Relevance


*University of Nebraska Medical Center, Omaha, Nebraska 68198; †Merck Research Laboratories, West Point, Pennsylvania 19486; ‡Pfizer, Inc., Groton, Connecticut 06340; §ILSI Health and Environmental Sciences Institute, Washington, DC 20005; ¶United States Environmental Protection Agency, Washington, DC 20460; ||Michigan Technology and Research Institute, Ann Arbor, Michigan 48104; |||Fulcrum Pharma Developments, Inc., Ann Arbor, Michigan 48103; |||National Institute of Environmental Health Sciences, National Toxicology Program, Research Triangle Park, North Carolina 27709; ††United States Food and Drug Administration, Silver Spring, Maryland 20993; **Indiana University School of Medicine, Indianapolis, Indiana 46202; and †††University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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Although rarely occurring in humans, hemangiosarcomas (HS) have become important in evaluating the potential human risk of several chemicals, including industrial, agricultural, and pharmaceutical agents. Spontaneous HS arise frequently in mice, less commonly in rats, and frequently in numerous breeds of dogs. This review explores knowledge gaps and uncertainties related to the mode of action (MOA) for the induction of HS in rodents, and evaluates the potential relevance for human risk. For genotoxic chemicals (vinyl chloride and thorotrast), significant information is available concerning the MOA. In contrast, numerous chemicals produce HS in rodents by non-genotoxic, proliferative mechanisms. An overall framework is presented, including direct and indirect actions on endothelial cells, paracrine effects in local tissues, activation of bone marrow endothelial precursor cells, and tissue hypoxia. Numerous obstacles are identified in investigations into the MOA for mouse HS and the relevance of the mouse tumors to humans, including lack of identifiable precursor lesions, usually late occurrence of the tumors, and complexities of endothelial biology. This review proposes a working MOA for HS induced by nongenotoxic compounds that can guide future research in this area. Importantly, a common MOA appears to exist for the nongenotoxic induction of HS, where there appears to be a convergence of multiple initiating events (e.g., hemolysis, decreased respiration, adipocyte growth) leading to either dysregulated angiogenesis and/or erythropoiesis that results from hypoxia and macrophage activation. These later events lead to the release of angiogenic growth factors and cytokines that stimulate endothelial cell proliferation, which, if sustained, provide the milieu that can lead to HS formation.

Key Words: hemangiosarcoma; angiogenesis; endothelial cells; endothelial precursor cells; mode of action; human relevance; PPAR agonists.

Although rarely occurring in humans (Weiss and Goldblum, 2008b), hemangiosarcomas (HS) have taken on significant importance in the evaluation of the potential for human risk of a variety of chemicals, including industrial, agricultural, and pharmaceutical agents. In contrast to humans, spontaneous HS are known to arise frequently in mice and less commonly in rats (Elwell et al., 2004; Haseman et al., 1998; Ruben et al., 1997). In addition, several breeds of dogs are known to have high spontaneous incidences of HS (Priester and McKay, 1980).

For DNA reactive, genotoxic chemicals such as vinyl chloride and thorotrast, which are known to induce HS in humans, significant information is known concerning the mode of action (MOA). In contrast, numerous commercial chemicals that produce HS in rodents act predominantly by non-DNA reactive, nongenotoxic, proliferative mechanisms. An overall framework is presented which includes several proposed MOAs involving various direct and indirect actions on endothelial cells. These include evidence for a possible role of adipose tissue as a source for endothelial growth factors for
agents such as peroxisome proliferator–activated receptor (PPAR) and retinoic acid receptor (RAR) agonists, a possible role for bone marrow–derived endothelial precursor cells in response to a net positive angiogenic stimulus, and a potential contributing role for hypoxia as a possible area for convergence across multiple MOAs (Figs. 1 and 2). Possible relationships between angiogenesis, a normal process in numerous biological phenomena, and vascular tumor induction are discussed, with many similarities identified but with evidence of significant differences.

The relevance of rodent hemangiomas and HS to human cancer risk is not always known. Vinyl chloride and vinyl bromide are known human carcinogens associated with HS, and also cause this type of tumor in rodents (Kielhorn et al., 2000; Morinello et al., 2002). Thus, it is reasonable to assume that when this tumor type occurs in rodents, it might be relevant to humans, unless there is evidence (i.e., a known MOA) to the contrary. There are likely to be several different pathways to HS following chemical exposure in rats and mice. Ultimately, these various pathways lead either to direct interaction with DNA and consequent DNA damage (DNA reactivity) or to an increase in cell proliferation which increases spontaneous mutations leading to an accumulation of cells that become dysfunctional with respect to the maintenance of mature differentiated endothelium and normal angiogenic processes (Cohen and Ellwein, 1991).

In contrast to the DNA reactivity and genotoxicity of vinyl chloride and vinyl bromide, most of the chemicals and pharmaceuticals associated with HS in rodents are non-DNA reactive (Nyska et al., 2004; Park et al., 2002). A preliminary examination of chronic rodent bioassays on pesticides show that increased incidences of HS occur relatively infrequently (approximately 3% of about 400 compounds evaluated in the rodent bioassay) (http://www.epa.gov/pesticides/carlist/). It is unclear if this small number might be due to the common use of 18-month bioassays in mice for pesticides in contrast to 24 months commonly used for pharmaceuticals. HS generally are observed late in a bioassay, that is, after 18 months, whether spontaneously or chemically induced. Given the small number of examples, it is difficult to make generalizations. However, this type of response does not appear to affect a particular chemical class of pesticides. Pesticides resulting in HS are generally not DNA reactive, although positive results in in vitro genotoxicity assays (e.g., clastogenicity) have been reported. Tumorigenic responses appear to be more common in the mouse compared to the rat, and most frequently occur in liver, spleen, heart, or subcutaneous adipose tissue. This tumor response is generally weak and sometimes not dose-related. MOA data are not typically available for pesticides.

Increased incidences of HS, primarily in mice, have been detected with a broad range of pharmaceutical and chemical agents (Table 1), including some calcium channel blockers, antipsychotics, PDE-5 inhibitors, DPP-4 inhibitors, antiarrhythmics, gonadotropin receptor antagonists, antisense compounds, nitric oxide releasers, hemolytic compounds, and vascular endothelial growth factor (VEGF) inducers (Jacobs, 2008).

Furthermore, HS in mice have been seen with many PPAR-γ and dual α/γ agonists which have led to restrictive measures
regarding clinical trials (EMEA, 2006a). Several companies have discontinued development of these drugs as a consequence. The occurrence of HS in mice with PPAR agonists and some other pharmaceutical classes has challenged regulators. During discussions on carcinogenicity by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), European regulators defended the approach that positive mouse carcinogenicity data did not necessarily lead to regulatory action (Van Oosterhout et al., 1997). The significance of PPAR-induced HS in mice is challenging because the human relevance of these mouse tumors remains unknown based on current knowledge gaps.

For pharmaceuticals, some aspects of the MOA may be associated with exaggerated pharmacological responses or off-target effects and may be relevant across species. However, MOAs may also be species specific due to (1) species differences in responsiveness to the chemical MOA, and (2) the interplay of this responsiveness with the genetic susceptibility factors governing the different spontaneous rates of HS development across species. Different classes of pharmaceuticals likely have different MOAs for formation of HS, but the MOAs may, especially in mice, have a common point of intersection and interaction with the growth regulatory pathway(s) that underlie the genetic predisposition to HS in this species. Thus, findings with pharmaceuticals complement what has been seen for other chemicals.

Understanding both the chemical MOA pathways to HS and any contributing species-specific susceptibility factors is essential to determine the relevance of these findings to humans. Analysis of MOA and human relevance is based on a framework developed over the past decade through the ILSI Risk Science Institute and the World Health Organization International Programme on Chemical Safety (Boobis et al., 2006, 2008; Meek et al., 2003; Seed et al., 2005; Sonich-Mullin et al., 2001). This review summarizes the pathology and biology of HS, discusses interspecies comparisons, compares and contrasts physiologic and pathologic angiogenesis, highlights examples of genotoxic and nongenotoxic compounds that induce HS, proposes an MOA framework for nongenotoxic compounds, and highlights the knowledge gaps. Importantly, despite the number of compound-specific initiating events (e.g., hemolysis, decreased respiration, adipocyte growth) (Table 1) that trigger nongenotoxic compounds to induce HS, there appears to be a common convergence, namely, dysregulated angiogenesis and/or erythropoiesis that results from hypoxia and macrophage activation. These later events lead to the release of angiogenic growth factors and cytokines that stimulate endothelial cell proliferation, which, if sustained, provide the milieu that can lead to HS formation.

FIG. 2. Proposed model for HS induction with comparison of phenotypic responses seen with two compounds that induce HS in mice, 2-BE (Laifenfeld et al., 2009) and pregabalin.
PATHOLOGY AND BIOLOGY

HS is an aggressive, malignant tumor of endothelial cells which is believed to arise from endothelium of blood vessels, and includes a variety of endothelial types, such as hepatic sinusoidal, venous, arterial, and capillary endothelia, or a bone marrow-derived stem cell (Weiss and Goldblum, 2008b). Because mature endothelial cells continue to proliferate in adulthood, they are at risk for neoplastic transformation. HS can arise in any organ, but are more commonly found in heart (right atrium), liver, spleen, lung, skin, soft tissues, mammary gland, and/or bone. Local invasion and metastasis are common, with metastasis frequently involving the lung and/or liver. Metastatic lesions can be difficult to distinguish from multicentrically arising HS.

Established causes in humans differ widely in pathogenetic mechanisms. Genotoxic mechanisms appear to account for liver HS induction associated with vinyl chloride exposures (197 cases, from Kielhorn et al., 2000) and thorotrast (29 cases, reported by Kojito et al., 1985), with median latencies of approximately 21 and 29 years, respectively, from onset of exposure to death. Breast angiosarcoma (Abbot and Palmieri, 2008) is associated with radiotherapy after breast-conserving therapy for breast cancer with relatively short latency (mean 72 months). However, in one study (Virtanen et al., 2007), breast and gynecological cancer patients were found to have a fivefold elevated risk of angiosarcoma compared to the general population, but the association with radiotherapy was not strong. Other etiologic factors in addition to radiation-induced DNA damage have been suggested, such as combined effects of radiation and chemotherapy, lymphedema associated with radiation and/or surgical treatment, and female hormonal factors (Abbot and Palmieri, 2008).

While HS are rare in humans (Weiss and Goldblum, 2008b), other vascular neoplasms are much more common (Weiss and Goldblum, 2008a). Kaposi’s sarcoma (Barillari and Ensoli, 2002), a related spindle cell sarcoma arising from lymphatic endothelium that forms vascular channels filled with blood cells, occurs at a 310-fold higher incidence than HS in the HIV-infected population. It is caused by human herpes virus 8 (HHV-8) infection, and is associated with elevated levels of cytokines (IL-1, IL-6, IL-8), HHV-8-encoded vIL-6, and other factors (leukemia inhibitory factor, oncostatin M, and cardiotropin) likely secreted by spindle cells and that act on gp130 receptors on endothelial cells (Amaral et al., 1993).

Other benign vascular neoplasms include pyogenic granulomas, cherry angiomas, infantile hemangiomas, and a variety of capillary and cavernous hemangiomas occurring most commonly in the skin and liver, but seen virtually in any tissue (Requena and Sangueza, 1997). Pyogenic granuloma is a relatively common benign vascular lesion of the skin and mucosa which is neither infectious nor granulomatous and

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<table>
<thead>
<tr>
<th>Chemical or drug class</th>
<th>Primary pharmacology</th>
<th>Potential MOA</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>2-Butoxyethanol</td>
<td>Industrial solvent</td>
<td>Hemolysis coupled with macrophage activation from iron accumulation leading to increase in ROS and local tissue hypoxia</td>
<td>Cormals et al., 2006</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>Insecticide—acetylcholinesterase inhibitor</td>
<td>Unknown—macrophage activation?</td>
<td>Bigot-Lasserre et al., 2000</td>
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<tr>
<td>Dronedarone</td>
<td>Antiarrhythmic</td>
<td>Unknown</td>
<td>EMEA, 2006b</td>
</tr>
<tr>
<td>Elmiron</td>
<td>Analgesic—relief of urinary bladder pain</td>
<td>Hemolysis leading to local tissue hypoxia</td>
<td>Abdo et al., 2003</td>
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<tr>
<td>Entecavir</td>
<td>Antivirals—nucleoside analogs</td>
<td>Unknown—PPAR-γ-like MOA</td>
<td>Physician’s Desk Reference, 2009</td>
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<tr>
<td>Fenretinide (4-hydroxyphenyl retinamide)</td>
<td>RAR agonist</td>
<td>Unknown—PPAR-γ receptor?</td>
<td>Cromwell and Goldenthal, 2006; Goodman, 1991</td>
</tr>
<tr>
<td>p-Chloroaniline, p-nitroaniline</td>
<td>Industrial solvents</td>
<td>Hemolysis leading to iron overload in macrophages and increased ROS</td>
<td>Nyska et al., 2004</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>δ28 subunit of voltage gated calcium—analgesic, anxiolytic, and antiseizure</td>
<td>Decreased respiration leading to local tissue hypoxia</td>
<td>Pegg et al., 2006; Physician’s Desk Reference, 2009</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>5-HT2/D2 antagonist—antipsychotic</td>
<td>Unknown</td>
<td>Physician’s Desk Reference, 2009</td>
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<tr>
<td>Troglitazone</td>
<td>PPAR-γ agonist—type II diabetes mellitus</td>
<td>Dysregulated angiogenesis leading to local tissue hypoxia</td>
<td>Herman et al., 2002</td>
</tr>
<tr>
<td>Tadalafil</td>
<td>PDE-5 inhibitor—erectile dysfunction</td>
<td>Unknown</td>
<td>Physician’s Desk Reference, 2009</td>
</tr>
<tr>
<td>Vildagliptin</td>
<td>DDP-4 inhibitor—type II diabetes mellitus</td>
<td>Dysregulated angiogenesis—inhibitor of angiogenesis</td>
<td>EMEA, 2007</td>
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whose exact cause is unknown. The lesion usually occurs in children and young adults as a solitary glistening red papule or nodule that is prone to bleeding and ulceration. Cherry angiomas are red papules on the skin containing an abnormal proliferation of blood vessels, and are the most common kind of angioma in adults. A small fraction have been linked to chemical exposures to mustard gas (Emadi et al., 2008; Firooz et al., 1999; Hefazi et al., 2006; Ma et al., 2006), 2-butoxyethanol (Raymond et al., 1998), bromides (Cohen et al., 2001), and cyclosporine (DeFelipe and Redondo, 1998). Hemangiomas are the most common benign tumor of infants, appearing usually within the first 2 weeks after birth, and up to five times more commonly in girls than boys (Weiss and Goldblum, 2008a). While 50% involute by age 5, regression takes longer for others and surgical treatment may be required. There is no evidence that this type of hemangioma progresses to HS.

There are also a number of hereditary syndromes in humans in which abnormalities of the vasculature and neoplasia of vessels or other tissues are both significant features, and for which an interrelationship between the changes leading to tumor formation in both epithelia and the vasculature are common and apparently genetically determined. These include (1) the syndromes (von Hippel-Lindau, Chuvash polycythemia) associated with mutations in the VHL gene which controls the stability of the hypoxia inducible factors (HIFs) regulating VEGF-mediated angiogenic responses (Foellmi-Adams et al., 2004; Hickey et al., 2007), and (2) the hamartomatous polyposis syndromes, Bannayan-Riley-Ruvalcaba (BRRZ) syndrome and Cowden syndrome (Pezzolesi et al., 2008; Rosner et al., 2008). VEGF-A, a specific endothelial cell mitogen, acts locally and systemically at tyrosine kinase growth factor receptors (VEGFR-1 and 2) to stimulate endothelial cell mitogenesis and recruit endothelial progenitor cells (Ferrara, 2002). Other factors with a predominantly local action in regulating angiogenic stimuli are platelet-derived growth factor, transforming growth factor β (TGFβ), fibroblast-derived growth factor (FGF), and hepatocyte growth factor (HGF) (Ferrara and Kerbel, 2005). The importance of VEGF signaling pathways in angiogenesis and HS is highlighted by the finding of mutations in the phosphatase and tensin homolog (PTEN) gene in canine and human HS; other common tumors such as endometrium, prostate, glioma, and melanoma; and 80 and 60% of patients with Cowden and BRRZ syndromes, respectively (Dickerson et al., 2005; Maehama et al., 2001). PTEN is a tumor and angiogenesis suppressor gene which inhibits angiogenesis due to its lipid phosphatase activity on substrates in the phosphatidylinositol-3 kinase pathway downstream from VEGF receptor signaling. Although these genetic disorders may shed light on vascular proliferation and angiogenesis, the vascular tumors associated with these are benign; HS are not a consequence of these disorders.

HS in rodent bioassays, either spontaneous or chemically induced, appear late in the 2-year studies. A major obstacle to the investigation of MOA for chemically induced HS has been the lack of a clearly defined, early precursor lesion. Although hemangiomas and HS are frequently combined in rodent bioassays for incidences of vascular lesions, hemangiomas are generally considered independent lesions that do not necessarily progress to HS in humans or animals (Weiss and Goldblum, 2008b). However, evidence exists that some lesions may be preneoplastic (i.e., angiomatous hyperplasia in the National Toxicology Program’s [NTP] butadiene study) and that occasionally HS are induced concurrently with or even arise within benign vascular neoplasms (Hardisty et al., 2007). In humans, there is no evidence that HS arise from hemangiomas or any other benign precursor (Weiss and Goldblum, 2008b).

The relationship of HS to other proliferative vascular changes may depend on a limited number of disturbances in the genetic control of physiologically essential angiogenic stimuli. Candidates for dysregulation include connective tissue growth factor (CTGF) and CD34. CTGF is a heparin-binding growth factor produced by fibroblasts after TGFβ activation that promotes angiogenesis in wound repair, tissue regeneration, and skin fibrosis (Igarashi et al., 1998). In mesenchymal tumors, CTGF mRNA was expressed in fibroblasts of dermatofibromas, some dermatofibrosarcomas, angiolipoma, angiomyleoma, and pyogenic granuloma, but not angiosarcoma, suggesting that benign fibroblasts and/or vascular endothelial cells have the capability to express CTGF mRNA when activated, but expression is lost in malignant tumors. Another candidate is the CD34 antigen (Lanza et al., 2001), a sialomucin glycoprotein involved in cell adhesion found to be expressed in dermotofibrosarcomas and angiosarcomas as well as other vascular proliferative lesions. It is also expressed in multipotent stromal cells (MSCs) from adipose tissue but not in bone marrow MSCs (Lazenec and Jorgensen, 2008). In summary, it is clear that a number of different potential pathways to angiosarcoma exist and that some assumptions that have been made about pathogenesis are unfounded.

**INTERSPECIES COMPARISONS**

A review of the spectrum of spontaneous and chemically induced vascular neoplasms in animals reveals important species and strain/breed differences in incidence, tissue distribution, tumor pathology, and susceptibility to tumorigenesis. Spontaneous HS are extremely rare (<0.001%) in humans (Weiss and Goldblum, 2008b); however, exposure-related incidences as high as 25% have occurred in humans occupationally exposed to vinyl chloride (Kielhorn et al., 2000). In contrast, HS are relatively common in domestic and experimental animals. The incidence in the dog is about 2%, and it varies by breed, with the German Shepherd dog and Golden Retrievers at greatest risk (Priester and McKay, 1980). HS spontaneously occur in about 0.1–2% of rats and 2–5% of mice (Elwell et al., 2004; Haseman et al., 1998; Ruben et al.,
than CBA/J.

For chemicals studied for carcinogenicity by the NTP (http://ntp.niehs.nih.gov/index.cfm?objectid=E8F394BD-1422-05ED-AD05E6097BA3D507), there have been 290 positive tests (“clear” or “some” evidence) in 550 studies in mice and rats, with 9% (25/290 studies) associated with induction of vascular neoplasms, and a few chemicals causing HS incidences of > 80% (Nyska et al., 2004). In only two of the 25 studies were vascular neoplasms the only neoplasm induced (4-chloro-o-toluidine and 2-biphenyl hydrochloride). In 19 of 25 studies, the vascular neoplasms were observed in mice only (10 in both sexes), in three of 25 studies in rat only, and in three of 25 studies, vascular neoplasms were seen in both mice and rats. The vascular system was a common site for chemically induced tumors, following liver, lung, mammary gland, hematopoietic system, forestomach, and thyroid. The overall incidences of hemangioma in control animals in NTP studies were 1 and 2% for male and female B6C3F1 mice and 0.3 and 0.25% in male and female F344 rats, with liver (mice), skin (mice and rats), uterus (mice and rats), and ovary (mice) being the most common spontaneous sites (Elwell et al., 2004; Nyska et al., 2004). For HS, the overall incidences in controls were 5% for male and female B6C3F1 mice and 0.5 and 0.4% in male and female F344 rats, with liver (mice), skin (mice and rats), spleen (mice and rats), bone marrow (mice), heart (mice and rats), uterus (mice and rats), and ovary (mice) being the most common spontaneous sites. For the B6C3F1 hybrid mouse strain used in NTP studies, there has been considerable variability in spontaneous incidence rates in different reports for hemangioma (1–15%) and HS (0–5%). The overall incidences of chemically induced HS in positive NTP studies were 22% (range 8–100%) and 25% (range 4–75%), respectively, for mice and rats. The five compounds with the highest reported incidence rates in NTP studies for HS (> 75% incidence) were riddelliine, 2-methyl-1-nitroanthroquinone, cupferron, tetrafluoroethylene, and o-nitrotoluene. The affected sites in positive studies were general/multiple sites, liver, spleen, skin/subcutis, abdomen/mesentery, heart, urinary bladder, and nasal cavity. Potential mechanisms that have been suggested include iron overload/hemosiderosis/hemolysis, hormonal perturbations, reduced antioxidant defense mechanisms, genotoxic events, induced effects on rates of cell proliferation and/or apoptosis, and dysregulation of cytokines and growth factors.

For riddelliine, a naturally occurring, genotoxic, pyrrolizidine alkaloid which induced a high incidence of hepatic HS in both rats and mice, short-term mechanistic studies (Nyska et al., 2002) showed reduced mitoses, increased hypertrophy and fatty degeneration in hepatocytes, with endothelial cells showing karyomegaly, cytomegaly, decreased apoptosis, more S-phase nuclei, and p53 positivity. Hepatocytes of treated animals expressed higher VEGF immunopositivity and altered endothelial cells staining positive for factor VIII–related antigen and VEGFR2. The authors concluded that HS development upon long-term exposure was likely promoted by induced changes in endothelial cell DNA adduct formation, apoptosis, proliferation of endothelial cells having undamaged and/or damaged DNA, and mutation rates. Further study (Moyer et al., 2004) indicated that damage to both hepatocytes and sinusoidal endothelial cells may lead to dysregulated VEGF synthesis by hepatocytes and activation of KDR/flk-1 by endothelium leading to the induction of sustained endothelial cell proliferation, culminating in the development of hepatic HS.

PAR-γ agonists are an example of nongenotoxic compounds associated with HS induction in mice (EMEA, 2006a). In 2006, a HESI PPAR Agonist Pathology Working Group convened to review diagnostic criteria for rodent proliferative mesenchymal lesions induced by this class of pharmaceuticals in rats and mice, and found that the spectrum of vascular lesions in mice included angiectasis, angiomatic hyperplasia, angiolipoma, hemangioma, and HS (Hardisty et al., 2007). With the exception of angiomatic hyperplasia and angiolipoma, the vascular neoplasms were similar in appearance to those described in publications by the Society of Toxicologic Pathology (STP) (Elwell et al., 2004; Ruben et al., 1997). Interestingly, a small number of cases was observed where HS was seen arising within angiolipomas. However, it remains unclear whether the angiolipomas were truly precursor lesions for the HS. In rats, PPAR-γ agonists did not induce vascular neoplasms but increased the incidence of lipoma, liposarcoma, fibrosarcoma, and mixed mesenchymal tumors, predominantly in the subcutis (Hardisty et al., 2007).

**Physiologic and Pathologic Angiogenesis**

Physiological angiogenesis is an essential part of many continuously running processes, including normal tissue development, wound healing, and endometrial cycling (Carmeliet, 2005; Ferrara and Kerbel, 2005). Vascular neoplasms are assumed to reflect aberrations in growth control of cells that are essential participants in normal vasculogenesis and angiogenesis. Physiologic angiogenesis is an intermittent, localized and tightly regulated response reflecting mainly an adaptive response to local hypoxia, and is dependent on the local accumulation of HIFs (Harris, 2002; Hickey et al., 2007). HIFs activate key angiogenic factors such as VEGF, whose pleiotropic effects include increased capillary permeability, stimulation of endothelial cell migration and proliferation, and the synthesis and release of the matrix metalloproteases (MMP-2) involved in matrix degradation. Downregulation of inhibitory factors such as angiopoietin 1 acting at the Tie2 receptor to maintain a quiescent state in mature endothelium is also required to allow angiogenesis to proceed. In contrast, pathologic angiogenesis as occurs in cancer, retinopathies, vascular
neoplasms, and other disease states is a sustained localized response characterized by tortuous, irregular, and leaky vessels. Activation of the “angiogenic switch” is thought to involve a balance between the growth factor activation pathways and a plethora of angiogenesis inhibitors such as thrombospondin-1, 16-kDa proctolin, interferon a/b, platelet factor-4, and angiostatin (Carmeliet, 2005; Ferrara, 2002; Ferrara and Kerbel, 2005; Harris, 2002; Kerbel and Folkman, 2002; Nyberg et al., 2005). Recent advances in our understanding of tumor angiogenesis have also highlighted the important roles of circulating, bone marrow–derived progenitor cells, including both endothelial and monocyte/macrophage lineage cells to pathological angiogenesis (Ding et al., 2008; Lamerato-Kozicki et al., 2006). These findings suggest that HS may arise not only from transformation of tissue-resident endothelial cell populations, but also from circulating progenitors or adult stem cells recruited from bone marrow or possibly also from extramedullary sites of hematopoiesis such as the liver and spleen.

The coordination of the recruitment, proliferation, localization, and differentiation of the different cell types that regulate angiogenic processes is exquisitely complex, involving many genes and offering a multitude of opportunities for genetic diversity (polymorphisms) to introduce species, strain, and individual animal variation in angiogenic responses. The genetic diversity in angiogenesis-regulating genes has been linked to increased susceptibility to multiple angiogenesis-dependent diseases in humans, including cancer (Rogers and D’Amato, 2006; Rogers et al., 2003, 2004). The spectrum of genetic differences may involve one or a few genetic loci, such as in Mendelian traits that are rare in the population, are minimally affected by environmental influences, and do not exhibit phenotypes in progeny that exceed the range of parental phenotypes. For example, in subsets of individuals with infantile hemangiomas (localized and rapidly growing regions of disorganized angiogenesis), missense mutations in the genes encoding VEGFR2 (KDR) and TEM8 (ANTXR1) result in suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling (Jinnin et al., 2008). Other examples include mutations in receptors in TGFβ/BMP signaling pathway genes (endoglin, ALK1) in hereditary hemorrhagic telangiectasias and Tie-2 mutations in venous malformations.

Genetic differences in angiogenic responses also result from complex traits affected by many loci that are common in the population and can be strongly affected by environmental influences. For example, polymorphisms in the human VEGF gene show significant associations with a variety of conditions and diseases (Rogers et al., 2004). Ongoing mapping studies (Rogers et al., 2003, 2004) have identified multiple loci that control angiogenic responsiveness in several mouse models such as the corneal micropocket and laser-induced choroidal neovascularization assays, raising the possibility that this approach may ultimately reveal genetic loci in mice that modulate susceptibility not only to angiogenic responsiveness but also to spontaneous and/or chemically induced HS. Genetic heterogeneity of the vasculogenic phenotype as measured by the number of circulating endothelial cells (Shaked et al., 2005) has also been shown to parallel angiogenic responsiveness in the corneal micropocket assay, suggesting this as a potentially useful surrogate clinical marker of angiogenic responsiveness that may have utility in predicting the progression of human angiogenic diseases.

Canine HS is reported to account for ~7% of dog cancers (Priester and McKay, 1980). Of the estimated 65 million dogs in the US, 1.5–2.5 million will likely be stricken. Because the tumor is poorly responsive to conventional therapy, most dogs will die from it within 1–8 months depending on the therapeutic modalities provided. A study of the molecular pathogenesis of canine HS showed no evidence of a canine gamma herpes virus or mutations in the ras or VHL genes. However, mutations in the C-terminal domain of PTEN were common, and may represent late events in tumor progression (Dickerson et al., 2005). Gene expression profiling clusters HS separately from nonmalignant (hemangioma) samples with enriched genes in HS in inflammation (IL-8, IL-6, PTGS2), angiogenesis and vessel response (VEGF-A, bFGF, ADM, PTGER4), adhesion (CD44, CDH2, FN1, NCAM1, VCAM1, ITGB3, and TNC), signaling/cell cycle/metabolism/mitosis (HGF, GUCY1A3, CDKN1a, CCND1, SLC2A3, SSP1), invasion (MMP-9, PLA2), and patterning (HOX-A10) (Tamburini et al., unpublished data).

Researchers in canine HS have also tested the hypothesis that genetic background (defined as “breed”) influences phenotypes and behavior of sporadic tumors. Results of this work have shown that tumor-restricted expression of proinflammatory and angiogenic genes in HS of Golden Retrievers cluster separately from HS of non-Golden Retrievers, suggesting that heritable factors mold phenotypes in sporadic, naturally occurring tumors. Gene sets enriched in Golden Retrievers compared to other breeds included genes overexpressed in plasmablastic cells (immune response), genes downregulated during differentiation of 3T3L1 fibroblasts into adipocytes with IDX (mesenchymal differentiation), genes upregulated in pulpal tissue from extracted cavities (inflammation), genes known to be induced by hypoxia (angiogenesis), and genes upregulated by HGF (endothelial growth response and survival) (Tamburini et al., unpublished data). Ontogeny analysis showed that 25/77-signaling pathways intersect at IL-8, and 6/77 pathways intersect at VEGFR1 (FLT-1) with recurrent enrichment shown for IL-1 response genes and acid ceramidase in Golden Retrievers with HS as compared to non-Golden Retrievers with HS. Future research directions include efforts to determine how VEGFR-1 and -2 activity and other metabolic pathways influence pathogenesis of canine HS and if canine HS may arise from a myeloid progenitor or, as previously suggested (Lamerato-Kozicki et al., 2006), from multipotential bone marrow–derived stem cells whose progeny arrest differentiation at the hemangioblast or angioblast stage.
Together, these data suggest that understanding the MOA and human relevance of chemically induced HS in rodents will need to consider not only the dose response relationships and chemical MOA for agents associated with HS induction, but also the intrinsic sensitivity and susceptibility of different rodent test species and strains and the genetic basis for these species differences.

VINYL CHLORIDE: A DNA REACTIVE CARCINOGEN

Vinyl chloride (VC) is a known animal and human carcinogen that induces HS of the liver as its hallmark cancer following inhalation exposure. In humans and animals, VC carcinogenesis is associated with relatively high exposures (≥ 50 ppm). VC is metabolized primarily by CYP2E1 to the epoxide, chloroethylene oxide, a highly unstable chemical that reacts with local cellular DNA and proteins, and is thought to have minimal systemic exposure. Four DNA adducts have been identified and have had molecular dosimetry studies conducted (Morinello et al., 2002). The major DNA adduct is 7-(2-oxoethyl) guanine (OEG), which comprises ~98% of the DNA adducts. This adduct is lost from DNA primarily by chemical depurination and is not considered to be promutagenic. Three etheno adducts are also formed and are thought to be promutagenic. All four of the DNA adducts of VC exhibit supralinear exposure response curves due to saturation of metabolic activation that results in a greater number of adducts being formed per ppm at lower exposures and plateauing of the exposure response at high exposures. Furthermore, four adducts are formed endogenously from lipid peroxidation products. Thus, there is always a background of identical endogenous adducts present. By exposing rats to [13C2]-VC, the half-lives of all four of the VC-induced adducts were determined. N\(^2\), 3-ethenoguanine (eG) has been shown to have a half-life of ~150 days, suggesting that it is very poorly repaired, if at all. In contrast, 1,N\(^6\)-ethenoxyadenosine (eDA) and 3N\(^4\)-ethenodeoxycytidine are actively repaired and have half-lives of ~1 day, while OEG has a half-life of ~4 days as a result of chemical depurination. Hepatic HS in animals and humans contain mutations in the p53 tumor suppressor gene and ras oncogenes that are compatible with the mutations caused by the three etheno adducts. The MOA for VC fits well with a mutagenic MOA.

However, recent investigations on a worker cohort in Louisville that was exposed to high concentrations of VC demonstrated that approximately 80% showed significant steatohepatitis that was not related to alcohol ingestion, diabetes, or obesity (Cave et al., 2008b). The 25 workers who developed HS also had VC-associated steatohepatitis, suggesting the possibility that it was a significant contributor to the pathogenesis of the HS, possibly contributing an increased proliferative stimulus to liver endothelial cells in which DNA mutation had occurred from the VC exposure. Release of a variety of cytokines, such as IL-1 and IL-6, from hepatocytes, could have provided such a stimulus. Because identical DNA adducts are produced endogenously that also occur after vinyl chloride exposure, risk assessments based on extrapolations to low-dose exposure are less clear. Furthermore, if steatohepatitis is required for the induction of HS by VC, it is likely to be a threshold toxicological phenomenon.

The recent investigations of the Louisville VC cohort also demonstrate that routine liver chemistries are not adequate as tumor markers for liver angiosarcomas, but preliminary data suggest that hyaluronic acid might be a reasonable early marker for future clinical development of HS (Cave et al., 2008a).

NON-DNA REACTIVE NONPHARMACEUTICAL CHEMICALS

Carbaryl induced an increased incidence of HS in both genders of mice but not in rats (US EPA, 2004). However, the increased incidence only occurred in mice administered carbaryl at a dietary dose of 8000 ppm which was in excess of the maximum tolerated dose based on decreased weight gain. Carbaryl was non-genotoxic in a battery of in vitro assays except for clastogenic activity in an in vitro Chinese hamster ovary assay with S9 activation. However, it was negative in an in vivo chromosomal aberration study, and also was negative when tested for carcinogenicity in the heterozygous p53 knockout mouse model.

2-Butoxyethanol (BE) has been reported to induce an increase in liver HS in male B6C3F1 mice following chronic inhalation, but not in female mice or in either gender in rats (Klaunig and Kamendulis, 2005). The mechanisms involved in BE-induced HS formation are not clear, but data suggest the involvement of oxidative damage subsequent to red blood cell (RBC) hemolysis and iron deposition and activation of Kupffer cells in the liver, events that exhibit a threshold in both animals and humans (Corhals et al., 2006; Klaunig and Kamendulis, 2005; Park et al., 2002). In isolated mouse and rat hepatocytes, neither BE or its major metabolite, 2-butoxyacetic acid (BAA), increased oxidative DNA damage (OH-8-dG), lipid peroxidation (MDA formation), or decreased vitamin E concentrations, while both ferrous sulfate (iron) and hemolyzed RBCs produced dose-related changes in biomarkers of oxidative stress (Klaunig and Kamendulis, 2005). Comparatively, mouse hepatocytes were more sensitive to oxidative stress by iron and hemolyzed RBCs when compared to the rat. In the Syrian Hamster Embryo cell transformation assay, BE and BAA did not induce cellular transformation. In contrast, iron produced dose-related increases in cell transformation, OH-8-dG, and DNA damage, effects that were prevented by coexposure to antioxidants (vitamin E or EGCG). In cultured endothelial cells, BE, BAA, and the aldehyde intermediate, butoxyacetaldheyde, did not induce DNA damage, using the single cell gel electrophoresis assay (COMET) (Corhals et al., 2006). However, iron, hemolyzed RBCs, and hydrogen peroxide increased DNA damage in endothelial cells.
Additional studies showed that activated macrophages increased both endothelial cell DNA damage and DNA synthesis, suggesting a role for macrophage activation in increased cell proliferation in endothelial cells (Corthals et al., 2006). Complementary *in vivo* studies have also been performed. In a subchronic study in mice and rats, BE induced hemolysis in both species at all time points (7, 14, 28, 90 days) and doses (225 and 450 mg/kg [rats]; 225, 450, and 900 mg/kg [mice]). Evidence of hemolysis was also shown by an increase in iron-stained Kupffer cells in both species, at the two highest doses of BE. Increases in OH-8-dG and MDA were biphasic and species selective: increases were seen after 7 and 90 days, selectively in mouse liver (Corthals et al., 2006). BE also produced a biphasic and species selective induction of DNA synthesis in mouse liver; endothelial cell DNA synthesis increased early after BE exposure (7 and 14 days), while DNA synthesis in hepatocytes increased only at 90 days.

To assess the role of Kupffer cells in BE-induced endothelial cell DNA synthesis, mice were given BE in the presence or absence of Kupffer cell depletions (via clodronate-encapsulated liposomes [CL]) for 7 days. BE increased the number of Kupffer cells (F4/80+ cells), while CL decreased Kupffer cell number by >90% (Corthals et al., 2006; Klaunig and Kamendulis, 2005; Park et al., 2002). BE produced the anticipated effects, including increased hemolysis, iron-stained nonparenchymal cells, and increased endothelial cell DNA synthesis. However, all values were similar to controls in Kupffer cell-depleted mice, suggesting that this cell type participates in BE-induced endothelial cell DNA synthesis. Collectively, evidence from *in vitro* and *in vivo* studies show that BE does not elicit direct effects on the liver, and provides suggestive evidence that the MOA for BE-induced liver HS involves indirect effects including hemolysis, Kupffer cell modulation, and oxidative stress (Klaunig and Kamendulis, 2005; Nyska et al., 2004).

### NON-DNA REACTIVE PHARMACEUTICALS

Several classes of pharmaceuticals have been associated with increased incidences of HS (Table 1). Three of these classes (retinoids, pregabalin, and PPAR agonists) have MOA information that has been published in the peer-reviewed scientific literature or elsewhere in the public domain and will be highlighted in this review.

Fenretinide is a prototypical retinoid which induces nearly a 100% incidence of HS at several sites in mice, but like the PPAR agonists, most are in adipose tissue, either subcutaneous or in the abdominal cavity (EMEA, 2006a; Herman et al., 2002; Johnson, 2008). Unlike many of the nongenotoxic pharmaceuticals associated with HS in mice, the tumors induced by fenretinide occurred earlier (e.g., as early as 1 year). These compounds bind the RAR, and evidence based on *in vitro* investigations demonstrates that they negatively regulate cell growth and angiogenesis, quite possibly through RAR-independent pathways. The issue of off-target receptor interaction is another of several critical issues that needs to be addressed in extrapolating the observations in mice to humans.

Endothelial cell apoptosis appears to be induced by fenretinide via increased production of ceramide by mechanisms not yet elucidated (Johnson, 2008). Fenretinide, like several other retinoids, inhibits the growth of a variety of neoplastic cell lines, including breast and oral cancers (Johnson, 2008). PPAR-γ transactivation is induced, as is increased nitric oxide production. No information is available as to whether these effects, particularly the association with PPAR-γ activation, are associated with the induction of HS. Likewise, two metabolites of fenretinide which do not bind to RAR have been identified, N-(4-methoxyphenyl) retinamide and 4-oxo-N-(4-hydroxyphenyl) retinamide, but their biological effects are mostly unknown.

Several possible factors have also been demonstrated to be involved in the HS induced in mice by pregabalin. Most of these tumors occurred in the tissues where spontaneous HS are commonly observed in mice, namely liver, spleen, and bone marrow (Criswell, 2008; Pegg et al., 2006). HS were induced in B6C3F1 and CD1 mice, but no tumors of any tissue were induced in rats.

The precipitating event in pregabalin-associated HS development in mice appears to be decreased respiratory function, including decreased respiration rate and decreased minute volumes, along with alterations in acid-base balance, gradually shifting to a relative alkalotic state (Criswell, 2008). Effects on respiration are also observed in rats administered pregabalin but, unlike mice, rats appear to adequately compensate with no resultant change in acid-base balance. The shift toward a more alkalotic state in mice, a species which normally has significantly more acidic blood than rats or humans, presumably could lead to decreased oxygen release from hemoglobin and subsequent local tissue hypoxia at high doses of pregabalin, initiating a cascade of events, including the overproduction of megakaryocytes and platelets, bone marrow congestion, macrophage infiltration and accumulation of iron on the macrophages with consequent release of various endothelial growth factors, including VEGF and PDGF and increased endothelial cell proliferation. However, the rats appear to adequately compensate whereas the mice do not.

### PPAR AGONISTS

Troglitazone is the prototypical low affinity (µM) PPAR-γ agonist developed to treat Type II diabetes. In carcinogenicity studies, it produced an increased incidence of HS in mice but not rats (Herman et al., 2002). These tumors were primarily seen in skin (usually in subcutaneous adipose tissue but included in skin histologic section) consistent with its pharmacologic action. The proposed HESI MOA framework...
for PPAR-\(\gamma\)-induced HS is presented below, using principally data from troglitazone, where appropriate, to illustrate these components. An outline of the framework is illustrated in Figure 1.

Troglitazone binds the PPAR-\(\gamma\) receptor, resulting in a conformational change that activates the transcriptional regulatory activity of the receptor and correlates with its antidiabetic actions (Berger et al., 1996). Troglitazone and several other PPAR-\(\gamma\)/PPAR dual agonists induce HS in mice (EMEA, 2006a; Herman et al., 2002). Muraglitazar, pioglitazone, and rosiglitazone also bind and activate PPAR-\(\gamma\), but do not produce HS; therefore, PPAR-\(\gamma\) binding appears to be necessary, but not sufficient, to produce HS (Pioglitazone SBA; Rosiglitazone SBA; Tannehill-Gregg et al., 2007; Waites et al., 2007). The reasons for induction of HS by some PPAR-\(\gamma\) agonists and not others are not known. Possible hypotheses include quantitative differences in receptor activation, differential recruitment of coactivators and corepressors, off-target receptor activities, or non-PPAR receptor activities, any of which could potentially impact cell cycle kinetics. Troglitazone and the other PPAR agonists are nongenotoxic in vitro and in vivo. The HS induced by troglitazone did not show evidence of mutations in p53 or ras genes, in contrast to what is frequently seen in human HS (Duddy et al., 1999a, 1999b).

Troglitazone induces an expansion of brown fat and promotion of brown adipocyte differentiation, increases adipocytes in Zucker rats, and promotes adipocyte differentiation of 3T3 fibroblasts in vitro (Okuno et al., 1998; Tai et al., 1996; Tafuri, 1996). Troglitazone-induced brown fat morphology shifts from a multinucleated to unilocular phenotype from upregulation of the mitochondrial uncoupling protein (UCP-1). Similar effects have been reported with pioglitazone (Foellmi-Adams et al., 1996; Sears et al., 1996). Hence, PPAR-\(\gamma\) agonists induce genes in adipocytes that drive differentiation and their growth.

Troglitazone induces HS in male and female mice in the 2-year bioassay at both 400 and 800 mg/kg/day (Herman et al., 2002). C-max at these doses is 79.9 and 86.9 \(\mu\)M, respectively. At the doses of 400 and 800 mg/kg/day in mice, there is an increased labeling index of endothelial cells in white and brown fat and in the liver after 4 weeks of administration, but not at 1 or 2 weeks (Kakiuchi-Kiyota et al., unpublished observations).

In a 2-week time-course experiment using BrdU administered via osmotic pumps, troglitazone, administered at a dose 50% greater than the highest dose used in the 2-year bioassay, increased adipocyte proliferation 3.9- and 1.5-fold at 1 and 2 weeks, respectively (Breider et al., 1999). Interscapular brown fat weight was increased at 2 weeks (1.21-fold), but not at 1 week (Breider et al., 1999). Troglitazone also produced a numerical increase in endothelial cell proliferation at 2 weeks (3.2-fold), but not at 1 week (Breider et al., 1999). These data demonstrate that adipocyte proliferation precedes endothelial cell proliferation, consistent with the proposed temporal linkage between adipogenesis and angiogenesis. Whether these temporal changes also occur at the same dose levels as the carcinogenicity study remains to be demonstrated.

Troglitazone was also evaluated for direct effects on endothelial cell cultures from microvascular endothelial cells obtained from skin and subcutaneous adipose tissue from mice (MFP MVEC) or humans (human microvascular endothelial cell [HMEC1]; Kakiuchi-Kiyota et al., 2009). The microvasculature of skin and adipose tissue was evaluated because of the predilection for PPAR agonist–induced HS to develop at these sites (Hardisty et al., 2007). In cytotoxicity experiments, the LD\(\text{50}\) for troglitazone on day 3 was 17.4 \(\mu\)M in HMEC1 and 92.2 \(\mu\)M for MFP MVEC cells. These concentrations are above the therapeutic blood level in humans and above the sarcomagenic blood levels in mice. Therapeutic blood levels in humans at an orally administered daily dose of 400 mg produces a C-max of 3.4 \(\mu\)M. Furthermore, there was no effect of troglitazone on viability of HMEC1 cells grown in medium with reduced growth factors, a standard method for assessing direct mitogenic effects. In contrast, troglitazone increased viability of the MFP MVEC cells at low concentrations, comparable to the blood concentrations of mice treated with sarcomagenic doses of troglitazone. The increased viability appears to be due to a combination of increased cell proliferation (assessed by \(^3\)H-thymidine incorporation) and decreased apoptosis (assessed by flow cytometry using Annexin V and propidium iodide staining). These in vitro studies suggest a fundamental difference between mouse and human endothelial cells in their biologic response to troglitazone. Similar studies with other PPAR agonists will ultimately be needed to fully compare and extrapolate results between mice and humans.

Adipocytes are a rich source of angiogenic growth factors (e.g., bFGF, VEGF); it is well recognized that adipogenesis is tightly linked with angiogenesis (Fukumura et al., 2003; Hutley et al., 2001; Kershaw and Flier, 2004; Rupnick et al., 2002; Sierra-Honigmann et al., 1998). Troglitazone-induced HS occur in highest frequency at sites associated with fat: adipose tissue, bone marrow, and skin/subcutis (Duddy et al., 1999a; Herman et al., 2002). There is some evidence that troglitazone and other PPAR-\(\gamma\) agonists increase angiogenic growth factors such as VEGF and possibly others. For instance, in vitro cultures of 3T3-L1 cells (an adipocyte differentiation model) demonstrated that pioglitazone and rosiglitazone produced smaller increases in VEGF levels and adiponectin than troglitazone, consistent with the ability of these agents to induce HS (Johnson, 2008). The relative contribution of angiogenic and/or inhibition of antiangiogenic growth factors (Nyberg et al., 2005) needs further study but is inhibited by the difficulty of these measurements. The large number of angiogenic and antiangiogenic growth factors precludes individual measurement but rather requires multiplex technologies and/or transcriptomic approaches to holistically assess the net angiogenic environment.
Dysregulated angiogenesis has been hypothesized as the basis for HS induction based on the following observations. PPAR-γ agonists stimulate adipocyte growth resulting in the release of angiogenic growth factors that stimulate angiogenesis to support the growth of the fat pad. In vitro data demonstrate that PPAR-γ agonists can inhibit endothelial growth (Bishop-Bailey and Hla, 1999; Murata et al., 2001). Hence, angiogenesis sufficient to support the growth of the fat pad is attenuated resulting in local tissue hypoxia. Alternatively, local tissue hypoxia may occur due to the inability of angiogenesis to keep pace with growth of the fat pad. The local tissue hypoxia results in HIF-1α activation which can activate VEGF secretion and/or macrophage activation. Macrophage activation leads to cytokine release (IL-1, IL-6), generation of reactive oxygen species, and inflammation (Corthals et al., 2006; Stienstra et al., 2008).

All of these changes can promote angiogenesis. Whether any of these relationships are also applicable to the induction of PPAR agonist–induced HS is unknown.

In addition to local stimulation of endothelial cell proliferation by angiogenic growth factors, an alternative hypothesis is that the target organ (i.e., fat pad in the case of PPAR-γ agonists) provides an angiogenic growth stimulus that recruits circulating endothelial progenitor cells (EPCs) from the bone marrow, and these stems cells seed the target organs leading to the formation of HS. The EPCs migrate to the target organ in response to a gradient of angiogenic growth factors. Rosiglitazone and pioglitazone have been shown to recruit EPCs to blood vessels or adipocyte tissue (Crossno et al., 2006; Werner et al., 2007); however, they do not induce HS. A potential differentiating factor may be that troglitazone also inhibits mitochondrial function unlike pioglitazone and rosiglitazone.

Sustained proliferation of endothelial cells can result in transformation from errors during DNA replication that are not repaired. In addition to this classical mechanism, there is growing evidence that hypoxia can drive and maintain genetic instability and a “mutator” phenotype (Bristow and Hill, 2008). Additional contributing factors for the transformation of endothelial cells include possible indirect DNA damage by macrophage activation and/or reduced antioxidant levels, as was shown with BE (Corthals et al., 2006).

**KNOWLEDGE GAPS**

In addition to the issues described above, the HESI MOA framework analysis for PPAR-γ–induced HS identified other knowledge gaps, several of which were discussed at the December 2008 Workshop. These knowledge gaps included the following:

- Data on endothelial cell proliferation using dual labeling methodology across PPAR-γ agonists;
- Absence of evidence for adipokine secretion, and more compelling data on the role of angiogenic/antiangiogenic growth factors;
- Assessment of the hypothesis that HS arises from recruitment of EPCs;
- Objective tools to identify preneoplastic lesions, if they exist;
- More data on species specificity of HS;
- In vitro comparative (mouse, rat, human) endothelial cell proliferation studies (the limitation is the difficulty in culturing sinusoidal-derived endothelial cells); and
- Pharmacogenomic assessment of mouse susceptibility to HS.

**SPECIES SPECIFICITY**

The species specificity of HS is not well understood, especially in the case of nongenotoxic compounds. There are several lines of evidence that suggest that the mouse is more susceptible to the induction of HS than humans:

1. Spontaneous incidence in mice is higher than in rats or humans, which suggests a genetic component as discussed in the Interspecies Comparisons section.
2. Spontaneous cell proliferation rates of endothelial cells across mice, rats, and humans correlate with the spontaneous incidence of HS (Ohnishi et al., 2007).
3. Troglitazone has been shown to stimulate cell proliferation in mouse microvascular endothelial cells but not HMEC (Kakiuchi-Kiyota et al., 2009).
4. Human disease states suggest that humans are more resistant than mice for development of HS. For instance, Chuvask polycythemia is a hypoxia-sensing disorder characterized by a homozygous mutation of the VHL gene (Gordeuk et al., 2004). These individuals have elevated levels of HIF-1α throughout their life, yet develop hemangiomas but not HS. In contrast, mice with a similar mutation in the VHL gene develop HS (Hickey et al., 2007).
5. Mice have lower antioxidant levels (e.g., Vitamin E) than rats and humans which may contribute to their enhanced susceptibility to developing HS (Corthals et al., 2006).

**SUMMARY**

In summary, HS induced by a variety of chemicals in rodent bioassays, particularly in mice, have increasingly become a significant issue with respect to regulatory decisions, especially for pharmaceuticals. A major difficulty in dealing with these rodent HS has been the lack of understanding of an MOA. Although significant factors in the MOA have been elucidated for DNA reactive carcinogens such as vinyl chloride, there are relatively little data available regarding MOAs for non-DNA reactive carcinogens inducing HS in...
mice, nor are there definitive data to address the issue of their human relevance. However, developments in basic biology, particularly related to research on angiogenesis, are providing significant advances in knowledge, and are beginning to identify possible MOAs and experimental approaches to addressing critical questions.

The MOA frameworks proposed in Figures 1 and 2 are the first report of a unified model for induction of HS by nongenotoxic compounds. Figure 1 summarizes the proposed MOA Framework for PPAR-γ agonist–induced HS. A critical component in this MOA is the concept of dysregulated angiogenesis which can result in local tissue hypoxia and the subsequent HIFα and/or macrophage activation. HIFα activation would lead to increased VEGF levels while macrophage activation would increase IL-6, both of which can stimulate endothelial cell proliferation. The concept of dysregulated angiogenesis has been proposed for troglitazone, fenretinide, and vildagliptin. Figure 2 proposes a general model for HS induction incorporating these concepts and compares the phenotypic responses seen with two other compounds that induce HS in mice, 2-BE and pregabalin. Both agents can induce local tissue hypoxia: 2-BE via its induction of hemolysis, and pregabalin via its respiratory suppression. Both compounds have been shown also to activate macrophages which may play a contributing role in the MOA. Hence, there do appear to be areas where divergent initiating events lead to a convergence around hypoxia and macrophage activation. Table 1 demonstrates that there are potentially multiple initiating events that lead to HS, while Figure 2 suggests that there may be convergence of these MOAs via dysregulated angiogenesis or erythropoiesis leading to hypoxia coupled with macrophage activation. This general model for HS provides the opportunity to test whether convergence does occur for other nongenotoxic compounds that induce HS.

Regarding human relevance, there appear to be significant differences between the responses of endothelial cells to various chemicals in different tissue sites, as well as between species. It remains unclear as to whether these responses represent sufficient qualitative differences between rodents and humans to impact the overall risk assessment for these chemicals. The only means to ultimately address the question of interspecies comparisons is to develop a better understanding of the MOAs leading to the development of these tumors, and to identify further biologic similarities and differences between rodents and humans.

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