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COMMENTARY

Mechanism of Action in Thalidomide Teratogenesis

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ABSTRACT. In this commentary, we describe a model to explain the mechanism of the embryopathy of thalidomide. We propose that thalidomide affects the following pathway during development: insulin-like growth factor I (IGF-I) and fibroblast growth factor 2 (FGF-2) stimulation of the transcription of αv and β3 integrin subunit genes. The resulting αvβ3 integrin dimer stimulates angiogenesis in the developing limb bud, which promotes outgrowth of the bud. The promoters of the *IGF-I* and *FGF-2* genes, the genes for their binding proteins and receptors, as well as the αv and β3 genes, lack typical TATA boxes, but instead contain multiple GC boxes (GGGCGG). Thalidomide, or a breakdown product of thalidomide, specifically binds to these GC promoter sites, decreasing transcription efficiency of the associated genes. A cumulative decrease interferes with normal angiogenesis, which results in truncation of the limb. Intercalation into G-rich promoter regions of DNA may explain why certain thalidomide analogs are not teratogenic while retaining their anti-tumor necrosis factor-α (TNF-α) activity, and suggests that we look elsewhere to explain the action of thalidomide on TNF-α. On the other hand, the anti-cancer action of thalidomide may be based on its antiangiogenic action, resulting from specific DNA intercalation. The tissue specificity of thalidomide and its effect against only certain neoplasias may be explained by the fact that various developing tissues and neoplasias depend on different angiogenesis or vasculogenesis pathways, only some of which are thalidomide-sensitive. BIOCHEM PHARMACOL **59**;12:1489–1499, 2000. © 2000 Elsevier Science Inc.

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Thalidomide is perhaps the most infamous drug in medical history [1–6], with possibly as many as 5000 papers having been published about the drug since its formulation in 1956 [7–9]. Recently, there has been a resurgence of interest in the drug because of its potential for treating a number of otherwise intractable diseases: ENL,† a complication of leprosy [10–12]; graft versus host disease [13–17]; weight loss in tuberculosis [18]; aphthous ulcers, wasting, and human immunodeficiency virus replication in acquired immune deficiency syndrome [19–21]; and cancer [22]. (For nearly 1500 references concerning the therapeutic potential of thalidomide, see Ref. 9.) With these new, expanding therapeutic uses for thalidomide, and in spite of the unprecedented precautions established by the Food and Drug Administration for the use of the drug, there are new risks of embryonic exposure and subsequent teratogenesis. As an example, a recent report listed 34 cases of thalidomide syndrome among children born between 1969 and 1995 in South America of mothers being treated for ENL [23]. This new risk of embryonic exposure heightens our need to identify the mechanism of the teratogenic action of the drug. By understanding the embryopathic mechanism of thalidomide, we may be able to develop a non-teratogenic analog of the drug to treat adult disease.

In spite of the fact that over 2000 papers have been published during the past 40 years specifically concerning thalidomide teratogenicity (out of the total of perhaps 5000), the teratogenic mechanism of action has remained elusive [24]. At least 30 hypotheses concerning the mechanism of action of this drug have been advanced. Stephens [24] reviewed 24 of those 30 hypotheses, 13 of which have been found to be incorrect; some of the others are supported by data, and yet others remain to be adequately tested. At least 6 additional hypotheses have been proposed since 1988. D'Amato *et al.* [22] have proposed that thalidomide is an angiogenesis inhibitor. Their hypothesis has received considerable attention in the past few years (thalidomide and angiogenesis will be discussed later in this paper). Neubert *et al.* [25] found that thalidomide can down-regulate certain integrins (thalidomide and integrins also will be discussed later in this paper). Oxidative DNA damage has also been presented as a possible mechanism of action [26, 27]. However, damage to DNA could result in mutations, and Ashby *et al.* [28] have demonstrated, in numerous test systems, that thalidomide is not mutagenic (the issue of oxidative DNA damage by thalidomide will be discussed in more detail later in this paper). It also has been suggested that the teratogenicity of thalidomide is mediated

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† *Abbreviations*: ENL, erythema nodosum leprosum; IGF-I, insulin-like growth factor I; FGF, fibroblast growth factor; Sp1, pregnancy-specific-β1-glycoprotein; AP1, activator protein 1; IGF-IR, insulin-like growth factor I receptor; AP2, activator protein 2; IGFBP, insulin-like growth factor binding protein; IRS-1, insulin receptor substrate-1; FGFR, fibroblast growth factor receptor; MKP, mitogen-activated protein kinase phosphatase; ERK, extracellular signal regulated kinase; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; and PDGF, platelet-derived growth factor.

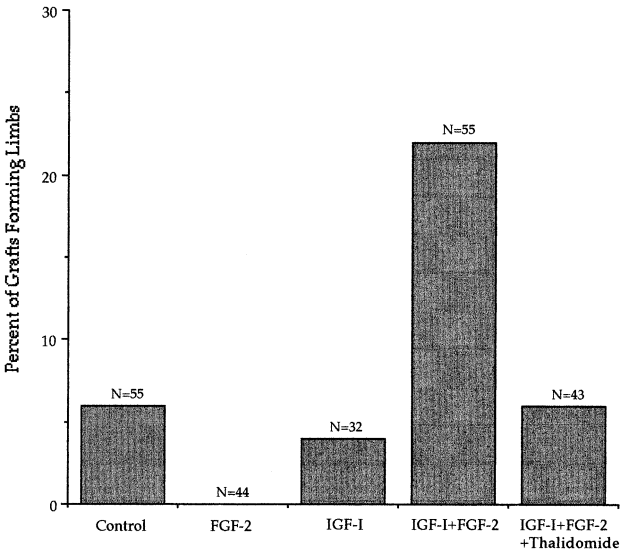

FIG. 1. Results of IGF-I, FGF-2, and thalidomide experiments conducted in our laboratory [33]. The future wing regions were removed from chick embryos of stages 11–12 [34]. The cut fragments were transferred to small petri dishes and incubated for 1 hr at room temperature in Ringer's solution only, or in Ringer's solution containing IGF-I (100 ng/mL, R&D), FGF-2 (100 ng/mL, R&D), and/or thalidomide (1–500 μg/mL, Grünenthal; Andrulis; Celgene). After incubation, the donor tissues were grafted to the celomic cavities of stage 18 host embryos *in ovo*. Then the host eggs were reincubated for an additional 6 or 7 days. Following the reincubation period, the embryos were fixed in 10% formalin and stained using standard techniques [35]. The embryos were evaluated for limb formation and were compared with control grafts exposed to Ringer's solution only. Control grafts formed limbs or limb-like growths in 6% of the cases. Wing territories exposed only to FGF-2 formed no limbs or limb-like growths. Wing territories exposed only to IGF-I formed limbs or limb-like growths in 4% of grafts. Wing territories exposed to IGF-I + FGF-2 formed limbs or limb-like growths in 22% of grafts. Wing territories exposed to IGF-I + FGF-2 + thalidomide formed limbs or limb-like growths in 6% of grafts. These data suggest that although neither IGF-I nor FGF-2 by itself can stimulate limb development in this experimental system, the two growth factors in combination are stimulatory. The data also suggest that thalidomide can inhibit the stimulatory effect of the combined growth factors.

by its anti-TNF-α activity [29]. However, it has been shown that the antiangiogenic activity of thalidomide correlates with its teratogenicity but not with its sedative or immunosuppressive properties [22], the latter of which are associated with serum TNF-α levels [11, 30], and that the antiangiogenic activity of thalidomide appears to be independent of its ability to suppress TNF-α production [31]. Another recent hypothesis is that thalidomide causes distalization of the embryonic limb bud without concomitant outgrowth of the bud [32]. Lastly, research in our laboratory suggests that IGF-I and FGF-2, in combination, are stimulatory to early limb development, and that thalidomide can reverse that stimulation [33] (Fig. 1).

The fifteen or sixteen proposed mechanisms that are plausible at the present time can be roughly grouped into

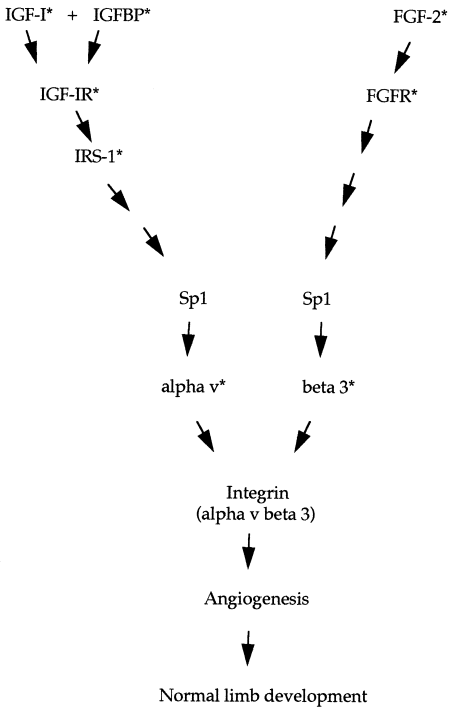

FIG. 2. Flow diagram demonstrating the proposed relationship between several proteins, whose syntheses are potentially sensitive to thalidomide, and angiogenesis and limb development. Abbreviations: IGF-I, insulin-like growth factor I; IGFBP, insulin-like growth factor binding protein; IGF-IR, insulin-like growth factor I receptor; IRS-1, insulin receptor substrate 1; FGF-2, fibroblast growth factor type 2; FGFR, fibroblast growth factor receptor; Sp1, pregnancy-specific-β1-glycoprotein; αv: integrin α subunit type v; β3, integrin β subunit type 3; and (*) genes with GGGCGG promoters.

six categories, with thalidomide affecting: (i) DNA replication or transcription, (ii) synthesis and/or function of growth factors, (iii) synthesis and/or function of integrins, (iv) angiogenesis, (v) chondrogenesis, or (vi) cell death or injury [1]. It may be that many of the remaining proposed mechanisms are correct and can fit into a unified model incorporating several of the mechanisms. We have proposed such a unified model [1] as follows (Fig. 2): we propose that IGF-I and FGF-2 synergistically stimulate integrin αvβ3 production by way of a promoter-specific transcription factor, Sp1, which binds to guanine-rich promoter regions of target genes. The *IGF-I* and *FGF-2* gene promoters, as well as those of their receptors, are also guanine-rich. The αvβ3 integrin, in turn, stimulates angiogenesis in the developing limb bud and a few other embryonic structures, such as the ear. Thalidomide affects this stimulatory pathway by binding to the guanine-rich promoters of the genes involved in the pathway, thus preventing the binding of Sp1 to the promoters and the transcription of the genes. This proposed model of thalidomide embryopathy unifies nearly all the previous models and provides biological relevance as well as biochemical and molecular specificity.

One of the proposed mechanisms of the teratogenic

action of thalidomide is that thalidomide intercalates into DNA [36]. Jönsson [36] proposed that a stacked complex is formed between the flat double phthalimide rings of thalidomide and deoxyguanosine. He cited data indicating that thalidomide in solution interacts with purines but not with pyrimidines, and that thalidomide has a greater affinity for guanine than for adenine. Considerable evidence, some of which is described below, supports the hypothesis that thalidomide intercalates into DNA [37–40].

Examination of a scale model of DNA (Carolina Biological, DNA model Kit B) and scale models of thalidomide and related models (such as hydrolysis products; cut from a second DNA model kit) reveals that there is not enough room for thalidomide or any of its primary metabolic products to intercalate into the minor groove of the DNA double helix because of the limited space between the nucleotides and the sugar-phosphate backbone. On the other hand, *S*-thalidomide, with its glutarimide moiety cocked slightly (about 30°) relative to the phthalimide plane, fits nicely into the major groove at purine sites if the glutarimide is directed toward the 3′ end of the sugar-phosphate backbone (Fig. 3). In this orientation, the oxygen of each of the carbonyl groups on the glutarimide ring is directed away from the phosphate backbone. However, *S*-thalidomide will not fit with the glutarimide directed toward the 5′ end because of spatial constraints between the carbonyl groups of the glutarimide ring and the phosphate backbone. *R*-Thalidomide, which is not teratogenic [41], also cannot fit with its glutarimide directed toward the 5′ end because of spatial constraints similar to those of *S*-thalidomide. Furthermore, when the glutarimide moiety of *R*-thalidomide is directed toward the 3′ end of the DNA molecule, the oxygen of one of the carbonyl groups on the glutarimide ring comes very close to the phosphate backbone. Therefore, *R*-thalidomide does not appear to fit into the major groove in any orientation.

Parman *et al.* [27] have recently presented data suggesting that the teratogenicity of thalidomide is associated with its ability to oxidize DNA. The specific oxidation they evaluated was at position 8 of deoxyguanylate, forming 8-hydroxy-2′-deoxyguanosine. Oxidation at position 8 should not be mutagenic, but oxidation at other positions on the guanine molecule (such as positions 1, 2, or 6) should be mutagenic, as they could interfere with hydrogen binding between base pairs across the center of the DNA molecule. Because thalidomide has been demonstrated not to be mutagenic [28], oxidation at those other positions of guanine apparently does not occur. Oxidation at position 8, which faces the open space of the major groove of the DNA molecule, may facilitate the intercalation of thalidomide into DNA at those sites. This may be especially true if the thalidomide molecule has been hydrolyzed so that the glutarimide ring is broken open (forming phthalimidoglutaramic acid). In this case, 8-hydroxy-2′-deoxyguanosine may provide a stabilizing influence on the intercalating

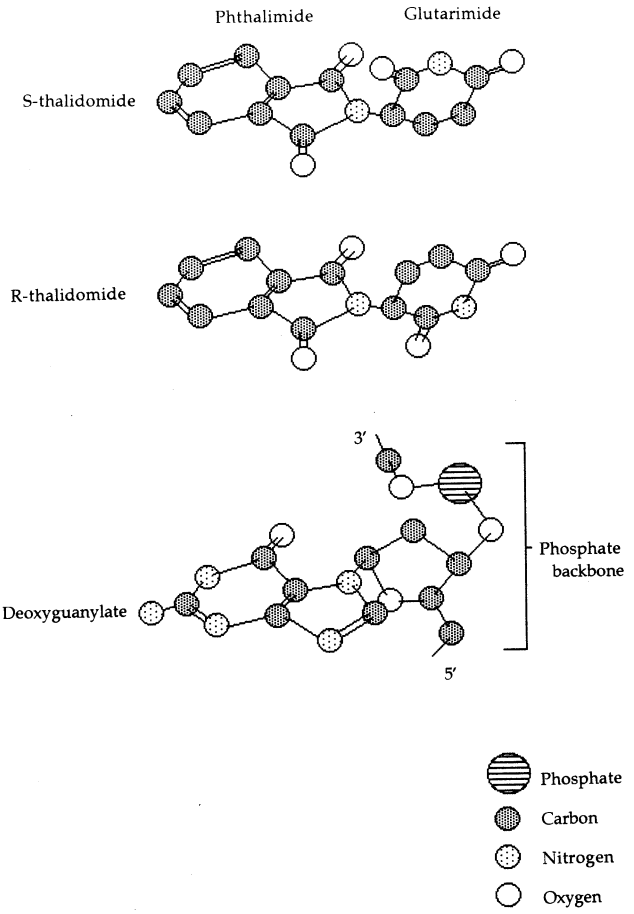

FIG. 3. Structures of *S*-thalidomide and *R*-thalidomide compared with that of deoxyguanylate. Note that the glutarimide portion of the thalidomide molecule protrudes out of the page toward the observer, whereas the sugar and phosphate backbone associated with deoxyguanylate recede into the page, away from the observer. Both carbonyl groups of *S*-thalidomide protrude away from the deoxyguanylate sugar and phosphate backbone, whereas only one carbonyl group of *R*-thalidomide protrudes away from the deoxyguanylate sugar and phosphate backbone and the other carbonyl group recedes back toward the sugar and phosphate backbone, coming into close contact with those parts of the DNA molecule.

thalidomide molecule or one of its hydrolysis products, such as phthalimidoglutaramic acid.

In spite of evidence supporting the DNA intercalation model of Jönsson [36], including that from molecular models, as described above, Jönsson's [36] model did not provide an explanation for the tissue or species specificity of thalidomide [8, 24, 42]. Without explaining the specificity of thalidomide, the biological significance of the model was lacking. We propose that expanding Jönsson's [36] model by incorporating recent molecular data provides the specificity missing in the original model.

The major teratogenic impact of thalidomide is on the limbs, ears, and eyes. If the intercalation of thalidomide at guanine-rich sites in DNA is key to its mechanism of action, such intercalation might have its greatest impact at the promoter regions of genes that are critical to the

development of the limbs, ears, and eyes. What, if anything, might be unique about the promoters of genes involved in the development of these structures? One answer may be that only 9% of gene promoters lack both a TATA and a CCAAT box, and rely instead on promoters with one or, more commonly, multiple GGGCGG sequences [43]. If thalidomide affects gene expression by intercalating into promoter regions, and if its intercalation is greatly enhanced by the presence of poly-G regions, then over 90% of all genes may be relatively unaffected by thalidomide intercalation. This concept alone may explain 90% of the specificity of thalidomide. We will discuss other issues later in this paper that could tighten this specificity further.

A promoter-specific transcription factor, called Sp1, which activates a class of promoters that includes the simian virus 40 early promoter and was first isolated from cell extracts by Dynan and Tjian [44], binds to the promoter hexanucleotide GGGCGG (the GC box) and belongs to a specific, novel subgroup of factors that are phosphorylated after binding to promoter sequences [45]. The zinc-dependent DNA binding region of Sp1 is localized in its carboxyl terminus, which contains three classical contiguous Cys_2-His_2 zinc finger domains [46]. The protein binds to B-DNA by docking in the major groove of the double helix, such that the protein wraps smoothly around the major groove, and each zinc finger makes contact with the G-rich strand, binding specifically to 5′-GNN-3′ triplets (N: any of the four nucleotides) within GC boxes [47–50]. Sp1f2 (zinc finger 2) contains two Arg residues spaced six residues apart, which recognize **GCG**, and Sp1f3 (zinc finger 3) contains an Arg and a His residue, spaced three residues apart, which recognize G**GG**. Polar side groups of these residues are located within hydrogen bonding distance of the 5′-phosphate in the DNA backbone and show interactions with the DNA backbone phosphates. These contacts with the DNA backbone apparently serve to position the Sp1 α-helix precisely within the major groove, and could play an important role in correctly docking and orienting the zinc fingers [46, 47].

Many of the promoters that lack TATA and CCAAT boxes but contain multiple Sp1-binding GC boxes are associated with "housekeeping" genes, which are constitutive, are transcribed at low rates in most tissues, and are subject to little, if any, regulation. However, Sp1 activity is not necessarily constitutive, but some Sp1 binding sites are involved in regulated promoter selection [44, 51]. For example, integrin gene expression may be facilitated by cooperative interaction between Sp1 and an AP1 binding site [52]. Furthermore, the binding of other factors to the promoter region may allow the general enhancement factor Sp1 to bind to the promoter in a tissue-specific fashion [53]. In at least some promoters, several tandem GC boxes (six or more) are lined up in the major groove of the DNA helix [54–56]. The various Sp1 binding sites of a single gene may be occupied at different times in development [56]. In addition, Sp1–Sp1 interactions may play an important role in modulating promoter activity [57], and there are also interactions between Ets-like proteins and Sp1 in transcriptional activation in TATA-less promoters [58]. Interestingly, the Sp1-binding site cannot be replaced by a functional TATA box in a TATA-less promoter [59]. Because most GC, Sp1 binding sites are constitutive and would not be expected to accommodate regulation during development, the number of G-rich promoters that could be affected by thalidomide during development is reduced further below the 90% reduction described above.

Intercalation of thalidomide into G-rich promoter domains would not appreciably affect promoters with the typical TATA and/or CCAAT transcription activator regions or G-rich promoters that either are constitutive or are not turned on during the period of exposure. However, intercalation of thalidomide into promoters that depend more heavily on actively regulated GC boxes could result in interference with normal gene function. If the affected genes are critical to some part of the developmental process, say for limb, eye, or ear development, such development may be compromised. Each step of a developmental pathway may not have to be affected to a great extent (perhaps as little as 10%) for the entire pathway to be profoundly affected.

It has been demonstrated that IGF-I plays an important role in embryonic development [60–62], including proliferation of early mesenchyme [63], lens cell growth and development [64], and angiogenesis [65–77]. IGF-I can also stimulate chondrogenesis and limb development [33, 68–70], and thalidomide can inhibit IGF-I stimulation of limb development [33]. The *IGF-I* gene promoter region has no TATA or CCAAT boxes, but is highly G-rich, with numerous Sp1 binding sites [78]. Therefore, the *IGF-I* gene promoter is a potential target for thalidomide intercalation.

FGFs also have been shown to play a significant role in limb development [79–89] as well as in limb initiation [77, 85, 90–96] and angiogenesis [22, 31, 97–99]. Further, there is evidence that limb initiation involves stimulation by a combination of IGF-I and FGF-2, and that thalidomide completely inhibits such stimulation [33, 77, 100, 101] (see Fig. 1). The *FGF-2* promoter region has no TATA or CCAAT boxes, but has multiple G-rich Sp1 and early growth response protein 1 (Egr-1) binding sites. The *FGF-2* gene, then, is another potential site of thalidomide interference in limb development. However, other FGF genes, such as *FGF-1* [102] and *FGF-4* [103], do have TATA and/or CCAAT boxes and do not depend as heavily on GC boxes for their function. As a result, other developmental processes, even those involved in limb development, such as apical ectodermal ridge function, may not be affected by thalidomide.

The actions of IGF-I on a given cell are mediated by activation of an IGF-IR, a transmembrane tyrosine kinase whose expression pattern is organ- and tissue-specific [104, 105]. It is expressed in the blastoderm of chick embryos during the first day of development, and is expressed continuously through most of development [62]. IGF-IR is autophosphorylated when it binds IGF-I and can, in turn,

phosphorylate other proteins within the cell [106]. The *IGF-IR* promoter region lacks both TATA and CCAAT boxes, but contains multiple Sp1 binding sites, as well as the AP2, electron-transferring flavoprotein, and Wilms' tumor suppressor binding sites [107–113]. Continuing a now familiar theme, here is another potential site of thalidomide interference. There is a strong correlation between the distribution of Sp1 and *IGF-IR* gene expression during development [109, 110].

The specific interaction of the IGFs with their receptors is facilitated by the presence of the IGFBPs [114]. IGFs usually form part of a protein complex with one of six IGFBPs, which can alter the transport and interactions of the IGFs with their receptors [67, 115–119]. At least one *IGFBP* gene promoter is TATA-less and contains three clustered GC box, Sp1 binding sites, as well as Sp1-related and other binding sites [120].

The principal cellular substrate phosphorylated by IGF-IR is IRS-1 [121–123]. The *IRS-1* gene promoter lacks typical TATA and CCAAT boxes but contains nine Sp1 binding sites [124]. IRS-1 contains at least 20 potential tyrosine phosphorylation sites. Once it is phosphorylated, IRS-1 can affect multiple regulatory pathways via signaling molecules containing src homology 2 domains (SH2 proteins), such as phosphatidylinositol-3-kinase [106, 122, 125–131].

The other initiating growth factor of this proposed pathway, FGF-2, binds to one of four FGFRs [132], which exhibit a remarkable degree of homology [133]. FGFRs are tyrosine kinases [134–136], whose genes lack the typical TATA and CCAAT boxes, but have GC islands with five classical Sp1 binding sites plus binding sites for transcription factors AP1, AP2, Krox-24, ornithine carbamoyltransferase 1, immunoglobulin heavy chain allotype C.4, GC factor, and Zeste [132, 135–139]. FGF-2 activates other proteins via a ras- and mitogen-activated protein kinase phosphatase (MKP2)-regulated pathway, a ras/raf-1/MEK/ERK-2/junD pathway, or a pathway converging upon a bipartite Ets-AP1 element [140, 141].

It has been shown that both IGF-I and FGF-2 can stimulate the production of the cell surface attachment integrin αvβ3 [98, 142, 143]. IGF-I stimulated a 2.4-fold increase in αvβ3 production in smooth muscle cells [143]. FGF-2 can stimulate a 4-fold increase in αvβ3 expression during enhanced angiogenesis on the chick chorioallantoic membrane [142]. The promoters for both the *αv* and *β3* genes lack the standard TATA and CCAAT boxes [144–147]. Rather, both promoters consist of G-rich promoters with multiple Sp1 binding sites and binding sites for other transcription factors (such as Ets, AP1, AP2, a κB-like motif, a vitamin D response element, and GATA-1) [144, 145, 147, 148]. At least one of these integrin subunits (β3) has been shown to be down-regulated by thalidomide [25]. Non-teratogenic thalidomide derivatives had no effect on adhesion molecule production [149]. Blaschuk *et al.* [150] demonstrated that down-regulation of αvβ3 could be mediated by regulating the expression of only the β3 subunit. However, with both αv and β3 dependent on GC promoter control, it is likely that thalidomide can affect the production of both subunits of the heterodimer.

Both IGF-I and FGF-2 have been demonstrated to stimulate angiogenesis [22, 31, 151–153]. A monoclonal antibody to αvβ3 blocks FGF-2-induced angiogenesis [142]. Thalidomide has been shown to be an inhibitor of FGF-2-stimulated angiogenesis [22, 98]. FGF-2-induced corneal neovascularization also can be inhibited by thalidomide [31].

Angiogenesis, the formation of new blood vessels from previously existing microvessels, is critical to embryogenesis, as is vasculogenesis, the *de novo* formation of vessels from endogenous angioblasts within a given tissue. Vasculogenesis and angiogenesis appear to be regulated by different, alternative mechanisms. For example, limb buds grafted to a celom are invaded by vascular endothelial cells from the host through the process of angiogenesis, whereas endothelial cells develop *in situ* within internal organs, such as the liver, grafted to a celom, through the process of vasculogenesis [154]. Pardanaud *et al.* [154] proposed that mesodermal/ectodermal associations stimulate angiogenesis, whereas mesodermal/endodermal associations stimulate vasculogenesis. This difference in developmental pathways may account for some of the tissue specificity of thalidomide.

Angiogenesis is stimulated by a variety of molecules, such as VEGF, FGF, TGF-β, PDGF, and others. VEGF and FGF act directly on endothelial cells, whereas TGF-β and PDGF attract inflammatory or connective tissue cells, which can stimulate angiogenesis ([98]; see also Refs. 99 and 155).

Several members of the integrin family are found at various sites on growing blood vessels. For example, α6 and β4 are found along the whole length of capillary loops, whereas α2 and αv are located mostly on the sprouts, and α5 is concentrated on the body of the vessel, away from the sprout [156]. αvβ3 and αvβ5 are both critical to angiogenesis [99, 155, 157–159], although αvβ3 has a primary role in most tissues [142, 159–165]. At least two cytokine-dependent angiogenesis pathways exist. FGF-2-stimulated angiogenesis depends on αvβ3, whereas VEGF-stimulated angiogenesis depends on αvβ5 [155]. Furthermore, diverse mechanisms may be involved in the stimulation of αvβ3 integrin expression in vascular endothelial cells [166]. IGF-I treatment of smooth muscle cells caused a 73% reduction in the α5 integrin subunit protein but a 25% increase in the αv subunit [143].

αvβ3 integrin stimulation may be involved in the angiogenic pathway employed by the developing limbs. This may account for the teratogenicity of thalidomide in the developing limb, and perhaps the ear, which may develop by a similar pathway. On the other hand, angiogenesis in the eye is stimulated by VEGF through the action of αvβ5 [167]. Kruse *et al.* [167] have shown that thalidomide can inhibit VEGF-αvβ5-stimulated angiogenesis. This may account for the teratogenic action of thalidomide in eye development. It is also of considerable interest that IGF-I

stimulates VEGF synthesis [151], and that the VEGF promoter is TATA-less, with Sp1, AP2, and nuclear factor-κB binding sites [168].

The interactions between integrins and the extracellular matrix have been identified as important regulators of vascular endothelial cell survival, proliferation, and migration during angiogenesis [169]. Extracellular matrix adhesion also is required in FGF-2 stimulated angiogenesis [97, 99, 170]. Ligation of the αvβ3 integrin on the surface of primordial capillary endothelial cells is critical for the differentiation and maturation of blood vessels, and it promotes a specific adhesion-dependent cell survival signal during angiogenesis, leading to specific suppression of endothelial cell death. Antagonists to integrin αvβ3 promote the unscheduled programmed death of endothelial cells in newly sprouting blood vessels [162, 165, 171].

Thalidomide apparently hydrolyzes rapidly in aqueous solutions with a pH above 6.0 [172]. The initial breakdown products are phthalimidoglutaramic acid (with the glutarimide ring open) and carboxybenzamidoglutarimide (with the phthalimide ring open). The data suggest that the phthalimidoglutaramic acids are teratogenic [173] and antiangiogenic [31], whereas carboxybenzamidoglutarimide is neither. These data agree with Jönsson's [36] model and our expansion of that model, suggesting that the intact phthalimide ring, which can intercalate into DNA at guanine-rich sites, is necessary for thalidomide antiangiogenesis and teratogenesis. It may be that phthalimidoglutaramic acid is a better intercalator than is the parent thalidomide molecule.

Examination of models suggests that carboxybenzamidoglutarimide (with the phthalimide ring open) does not orient well with the purine nucleotides. On the other hand, phthalimidoglutaramic acid (with the glutarimide ring open) appears to have even more room relative to the phosphate backbone than does the closed glutarimide ring of the native thalidomide molecule. EM-12, which is a more potent teratogen than thalidomide [25, 174], has one carbonyl group removed, allowing even more room for the total molecule relative to the sugar of the DNA backbone. On the other hand, it is not clear from molecular models alone why EM-16 is not teratogenic.

Bioactivation (or bioinactivation) of thalidomide or its hydrolysis products by microsomes results in five primary metabolites, two of which are hydroxylated on the phthalimide moiety and three of which are hydroxylated on the glutarimide moiety [175]. Whereas most data suggest that bioactivation of thalidomide is necessary for its teratogenic action (cf. Ref. 175), other data suggest that hydroxylation is inactivating [31]. While it is clearly important to discover which of the numerous hydrolysis and metabolic products of thalidomide are teratogenic, our model is not altered appreciably by the presence or absence of hydroxyl groups on the molecules. Such modification, especially to the phthalimide ring, would make the molecule more hydrophilic and may enhance either clearing through the kidney (inactivation) or delivery to the embryo (activation).

Establishment of the teratogenic action of thalidomide as based on its ability to intercalate into guanine-rich promoter regions of the DNA has significant implications for future research. For example, such information can help explain why certain amino-substituted thalidomide analogs are not teratogenic while remaining potent inhibitors of TNF-α production [176]. This knowledge also suggests that we look in some direction other than intercalation into DNA to explain the action of thalidomide on TNF-α. On the other hand, the DNA intercalation of thalidomide is probably the basis of its anti-cancer action, by way of its antiangiogenic action. However, various neoplasias may depend on different angiogenesis or vasculogenesis pathways, which may explain why some cancers are thalidomide sensitive whereas others are not. Furthermore, it also would be of considerable interest to determine the mechanism by which thalidomide causes peripheral neuropathy. Is this mechanism related to its intercalation or to some other mechanism, perhaps more related to its effect on TNF-α?

One of the major questions still remaining unresolved is whether the action of thalidomide is via the native molecule or some hydrolysis or metabolic product. It is also of great interest to determine how the interaction of these molecules with DNA could be enhanced by DNA oxidation, especially at position 8. These and other, related questions will provide several exciting topics for research extending into the future.

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