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Editorial

A Novel Hypothesis for Thalidomide-Induced Limb Teratogenesis: Redox Misregulation of the NF-κB Pathway

JASON M. HANSEN[1] and CRAIG HARRIS[2]

ABSTRACT

Several hypotheses have been proposed to explain the mechanisms of thalidomide teratogenesis, although none adequately accounts for the observed malformations and explains the basis for species specificity. Recent observations that thalidomide increases the production of free radicals and elicits oxidative stress, coupled with new insights into the redox regulation of nuclear transcription factors, lead to the suggestion that thalidomide may act through redox misregulation of the limb outgrowth pathways. Oxidative stress, as marked by glutathione depletion/oxidation and a shift in intracellular redox potential toward the positive, occurs preferentially in limbs of thalidomide-sensitive rabbits, but not in resistant rats. DNA binding of nuclear factor κ-B (NF-κB), a redox-sensitive transcription factor and key regulator of limb outgrowth, was shown to be significantly attenuated in rabbit limb cells and could be restored following the addition of a free radical spin-trapping agent, phenyl *N-tert*-butyl nitrone. The inability of NF-κB to bind to its DNA promoter results in the failure of limb cells to express fibroblast growth factor (FGF)-10 and twist in the limb progress zone (PZ) mesenchyme, which in turn attenuates expression of FGF-8 in the apical ectodermal ridge (AER). Failure to establish an FGF-10/FGF-8 feedback loop between the PZ and AER results in the truncation of limb outgrowth. We hypothesize that species-selective alterations in redox microenvironment caused by free radical production from thalidomide results in attenuation of the NF-κB-mediated gene expression that is responsible for limb outgrowth. *Antioxid. Redox Signal.* 6, 1–14.

INTRODUCTION

THALIDOMIDE (α-PHTHALIMIDOPHTHALIMIDE) was first introduced in 1957 as a mild sedative–hypnotic and was successfully distributed in several countries under numerous trade names. The list of indications was expanded as additional therapeutic uses for the drug were discovered. A lack of deleterious side effects, the inability to determine lethal concentrations, and the efficacy to relieve nausea associated with pregnancy resulted in increased use by pregnant women. Nearly 4 years after its introduction to the public, thalidomide was found to be responsible for a broad spectrum of birth defects, the most notable being limb reduction defects (phocomelia, amelia). The drug was subsequently removed from the market because of the birth defects and manifestations of an often irreversible peripheral neuropathy seen in adult patients who had taken thalidomide for longer periods of time (17, 28, 52, 89). Although research was initially intense in attempts to understand mechanisms of thalidomide-induced limb dysmorphogenesis, no clear mechanisms were found, and efforts waned due to thalidomide no longer being a threat to public health. Interest in understanding the mechanism of thalidomide-induced teratogenesis has been rekindled, however, because the drug was recently approved for sale and use in Europe and South and North America. It is currently being used as therapy for a variety of diseases, such as erythema nodosum leprosum, human immunodeficiency virus-related wasting syndrome and esophageal ulcers, graft-versus-host disease, arthritis, and tuberculosis and is being considered and tested as a therapy for a number of other diseases (13, 56, 63).

[1]Department of Biochemistry, Emory University, Atlanta, GA.
[2]Toxicology Program, Department of Environmental Health Sciences, University of Michigan, Ann Arbor, MI.

Reintroduction of thalidomide for general medicinal use renews the possibility of thalidomide-induced terata despite efforts to regulate its application and to inform the public about the potential dangers of misuse.

In all, >30 hypotheses have been proposed to explain mechanisms of thalidomide teratogenesis. The significant hypotheses, presented to date, that are still under consideration are summarized in Table 1. Many have focused on disruption of biochemical and molecular pathways and include DNA intercalation, acetylation of macromolecules, interference of glutamate metabolism, and folic acid antagonism (5, 25, 40, 45, 79). Two recent hypotheses have suggested that thalidomide-induced disruption of angiogenesis and the decreased expression of adhesion receptors are also possible teratogenic mechanisms (20, 62). Stephens and Fillmore (80) hypothesized that thalidomide may interfere with the promoter of specific genes [*Igf-1* (insulin growth factor-1) and *Fgf-2* (fibroblast growth factor-2)] involved in limb outgrowth and development, suggesting that thalidomide intercalates into DNA regions that are guanine- and cytosine-rich, decreasing gene expression and resulting in limb truncation (83). Still, a paucity of experimental evidence exists to either support or refute many of these mechanisms. For some hypotheses, there has been experimental support, but no single mechanism was elucidated, and sufficient evidence is unavailable to adequately explain thalidomide-induced limb reduction and account for species specificity.

Here, we describe a novel molecular mechanism by which thalidomide causes limb reduction defects.

Hypothesis: *Thalidomide causes oxidative stress and a change in intracellular redox potential in limb progress zones where species-specific redox environments are not intrinsically or rapidly restored to reducing conditions. Oxidative redox potentials, particularly in the nucleus, cause* S*-glutathionylation of nuclear factor-κB (NF-κB) and prevent NF-κB binding to DNA. The subsequent NF-κB-dependent gene expressions that are responsible for maintaining limb outgrowth are attenuated, producing phocomelia and amelia in affected offspring.*

THALIDOMIDE INCREASES FREE RADICAL PRODUCTION AND ELICITS OXIDATIVE STRESS

Metabolic biotransformation of thalidomide has been at the center of a long-standing debate surrounding the mechanisms of thalidomide teratogenicity. Addition of thalidomide to biological fluids results in the rapid, and presumably nonenzymatic, hydrolysis to a number of ring-opened and truncated products. The identity of the ultimate teratogenic species, however, remains undetermined. Some of the biological and therapeutic actions of thalidomide have now been shown to require its metabolic biotransformation (8, 58). Reports have provided evidence that prostaglandin H synthase may be capable of bioactivating thalidomide (4), and new evidence implicates a cytochrome P450 isozyme, CYP1A1, as the bioactivator that produces decreases in fibroblast proliferation (58). Demonstration that an increased toxicity is seen with the S-isomer of thalidomide suggests that at least one macromolecular target is involved in its mechanistic interactions (65, 66). It is not known whether the stereoselectivity is related to a transport step, a signaling step, a biotransformation step, or any other interaction.

TABLE 1. ACTIVE HYPOTHESES TO EXPLAIN THE MECHANISM OF THALIDOMIDE (1966–2003)

Hypothesis	*Authors*	*Reference*
Acylation of macromolecules	Jonsson (1972)	40
Ascorbic acid synthesis	Vaisman *et al.* (1983)	87
DNA intercalation	Jonsson (1972)	40
	Stephens and Fillmore (2000)	80
Disruption of angiogenesis	Jurand (1966)	42
	D'Amato *et al.* (1994)	20
	Sauer *et al.* (2000)	74
Down-regulation of adhesion receptors	Neubert *et al.* (1996)	62
Alteration of cytokine synthesis (tumor necrosis factorα)	Sampaio *et al.* (1991)	73
Folic acid antagonism	Kemper (1962)	45
Inhibition of DNA synthesis	Bakay and Nyhan (1968)	5
DNA oxidation	Parman *et al.* (1999)	68
Interference of glutamate metabolism	Faigle *et al.* (1962)	25
Mesonephros-stimulated chondrogenesis	Lash and Saxen (1971)	50
	Lash and Saxen (1972)	51
	Stephens and McNulty (1981)	81
	Stephens and Pugmire (1986)	82
Oxidative stress	Hansen *et al.* (1999)	32
	Hansen *et al.* (2002)	33
	Hansen *et al.* (2002)	34
	Parman *et al.* (1999)	68
	Sauer *et al.* (2000)	74

A significant breakthrough in our understanding of thalidomide's effects has come with the observation that thalidomide acts as an oxidant and modulator of intracellular redox potential. Sauer and co-workers (74) confirmed that thalidomide forms hydroxyl radicals in murine embryonic stem cells, a pluripotent cell line that can be induced to form embryoid bodies, which differentiate to contain many different cell types, including vascular structures. Thalidomide significantly reduced angiogenesis in treated embryoid bodies, which was correlated with the production of reactive oxygen species (ROS). Addition of the free radical scavengers, 2-mercaptoethanol and mannitol, completely abolished thalidomide-induced antiangiogenic effects, further supporting a free radical-based mechanism for inhibition of angiogenesis by thalidomide (74). A previous report by Bauer *et al.* (8) provided evidence that metabolic bioactivation of thalidomide was necessary for its ability to inhibit angiogenesis. They further showed that microsomes from sensitive species, such as the rabbit and the human, are capable of activating thalidomide to promote inhibition of angiogenesis, whereas rat microsomes were ineffective.

Wells and co-workers (68) compared effects in two species, thalidomide-sensitive rabbits and thalidomide-resistant mice, in experiments designed to understand whether the species differences seen in response to thalidomide could help elucidate possible teratogenic mechanisms. Rabbits were treated with 400 mg/kg/day thalidomide and produced litters with limb malformations (phocomelia) and other expected thalidomide-related defects (omphalocele). Thalidomide-exposed fetuses had considerably higher concentrations of oxidized DNA based on measurements of enhanced 8-hydroxy-2′-deoxyguanosine formation, a marker of oxidative stress, as compared with control fetuses. In thalidomide-resistant mice, litters showed no significant increases in limb malformation or DNA oxidation, even with thalidomide doses as high as 1,600 mg/kg/day. These results suggest that rabbit, but not mouse, fetuses accumulate damage due to oxidative stress, and that there are inherent factors particular to the rabbit that allow this to occur. Rabbits receiving thalidomide treatments were also cotreated with a free radical spin-trapping agent, α-phenyl-*N*-*tert*-butylnitrone (PBN), to determine whether the direct removal of free radicals prevented the observed toxic outcome. PBN coadministration significantly decreased limb malformations and, also, decreased DNA oxidation compared with rabbit fetuses exposed to thalidomide only *in utero*. Wells and co-workers suggest that thalidomide embryopathy involves free radical-mediated oxidative damage to embryonic DNA (68).

By using a similar comparative approach, rat and rabbit limb bud cells (LBCs) were collected at similar developmental stages (based on embryo somite number), grown in culture, and treated with thalidomide *in vitro*. The production of ROS was determined using dichlorofluorescein (DCF), a dye that produces a stable fluorescent product upon interaction with hydrogen peroxide. Rat LBCs treated with 100 μ*M* thalidomide produced very little increase in DCF fluorescence (~40%), but rabbit LBCs treated with the same concentrations increased by nearly 200% from basal levels, approximately fivefold greater than that seen in the rat. Treatment with PBN attenuated thalidomide-induced ROS production in both rat and rabbit LBCs as indicated by a return of DCF fluorescence to baseline levels (34). These results suggest that thalidomide directly increases concentrations of ROS in cultured limb cells and that the rabbit LBC is more apt to accumulate ROS than is the rat LBC. Rat and rabbit LBCs also showed evidence of ROS production following thalidomide exposure based on the colocalization of redox-sensitive dyes to mitochondria (34).

Although the above studies (34, 68, 74) implicate the generation of free radicals in thalidomide-induced limb teratogenesis, they do not resolve the mechanism by which the free radicals cause specific alterations of limb bud growth and development. It has been suggested that an abundance of ROS may create a permissive oxidative environment that allows for signal transduction to be stimulated or inhibited, depending on the initiating signal (75). The cellular response to ROS production involves attempts to remove free radicals directly or indirectly through the reduced glutathione (GSH)-dependent detoxication pathways. As GSH is oxidized to glutathione disulfide (GSSG) in the detoxication processes, ratios of GSH/GSSG decrease, leading to a shift in the intracellular redox potential. Redox potentials are calculated from measured concentrations of GSH and GSSG using the Nernst equation (75). GSH is the most ubiquitous antioxidant, accounting for nearly 90% of all intracellular reducing equivalents found in cells (18) and is crucial in the maintenance of intracellular redox potential (75). As redox potential is modulated by ROS formation, a number of different cellular processes related to metabolic regulation, signaling, and gene expression can be inhibited or activated in response to oxidative changes to the intracellular environment and may correlate with the promotion or inhibition of proliferation, differentiation, and apoptosis (1–3, 9, 10, 12, 18, 36, 39, 46, 61, 77, 91). Maintenance of the proper redox potential may prove to be even more critical during development as cell populations are actively participating in proliferation, differentiation, and apoptosis at various rates during critical periods of initial growth. Chemically induced redox changes could promote untimely activation or inhibition of developmental pathways, resulting in faulty development and teratogenesis.

Initiation of conditions characterized as oxidative stress occurs when increases in reactive species result in the net oxidation and depletion of GSH to a level where intracellular redox potential is shifted to the positive and cellular damage ensues. The ultimate manifestation of damage and duration of altered redox potential is determined by the host's inherent ability to restore GSH redox status through disulfide reduction and *de novo* synthesis. Hansen and co-workers (32) used two species with markedly different sensitivities to thalidomide, the resistant Sprague–Dawley rat and sensitive New Zealand White rabbit, to show that thalidomide exposure in whole embryo culture results in a significantly greater GSH depletion in the rabbit than in the rat conceptus over a 24-h period. As GSH is the single largest contributor to the intracellular pool of reducing equivalents (18), these results confirm the preservation of a species sensitivity to thalidomide *in vitro* and also implicate a deficiency in the rabbit protective systems that results in a greater redox shift than seen in the rat embryo under similar conditions of oxidative insult.

Rat LBCs contain ~50% more GSH than rabbit LBCs (32), suggesting a greater protective effect in the rat than the rabbit LBC during periods of oxidative stress. To a greater extent, similar species-specific trends were also evident with cysteine, which represents another important pool of reducing equivalents and a critical rate-limiting precursor amino acid required for *de novo* GSH synthesis. Rat LBC cysteine concentrations were nearly 3.5-fold greater than those in rabbit LBCs, suggesting that the rat is more capable of handling a higher oxidative load than the rabbit and is more capable of a more complete restoration of GSH by *de novo* synthesis following depletion.

Additional comparisons of GSH status show that rat embryos contain nearly 35% more GSH, a difference that contributes to a +10 mV difference in redox potential between the rabbit (−204 mV) and rat (−214 mV) embryos. Specific redox differences within the limb followed similar trends, with an +11 mV difference between untreated rabbit and rat limbs, measuring −164 mV in the rabbit limb and −175 mV in the rat limb. Findings indicate that the limbs of both species are highly oxidative as compared with other embryonic tissues (*i.e.,* trunk and head), the overall embryonic redox potential is less negative (more oxidative) in the rabbit than the rat, and the rabbit limb is much more oxidative than the rat limb.

Another critical redox couple involved in the regulation of several cellular pathways is the thioredoxin (Trx) and thioredoxin reductase redox couple. This enzyme pair has been shown to be essential for the maintenance of a number of active-site protein thiols (transcription factors) in their reduced and active states. Differences in limb Trx were significant between the sensitive and resistant species, where the rat limb had sixfold greater Trx concentrations than did the rabbit limb. These differences were not seen in the other regions of the embryo proper. Significant differences in Trx could suggest at least three major regulatory and functional implications for developmental outcome. First, Trx stimulates and interacts with other antioxidant defense systems (21) and can reactivate oxidized proteins following their deactivation by hydrogen peroxide and other ROS exposures (26). Second, Trx acts as an antioxidant itself, interacting directly with other disulfides to facilitate their reduction. Trx can also interact directly with ROS, acting as a proton donor, dimerizing and removing intracellular ROS in the process. Third, Trx may be involved in protein–DNA interactions. Many transcription factors are redox-sensitive in that they contain sulfhydryls (cysteine residues) in their DNA binding domains. These cysteine residues must be maintained in a reduced state in order for productive binding and subsequent gene transactivation to occur (22, 29, 37). Because there is inherently less Trx in the rabbit limb, the Trx-mediated (de)activation of specific enzymes or transcription factors may be inhibited or diminished to a greater degree than in the rat limb.

We have measured inherent differences in limb redox potentials, limb Trx, GSH, and cysteine concentrations between the rat and rabbit, suggesting that the rat limb is less sensitive to redox misregulation because it maintains a much more reducing environment, higher Trx concentrations to regulate Trx-mediated processes, and higher GSH and cysteine stores to buffer redox potentials altered by ROS. Conversely, the rabbit limb maintains a more oxidative environment, contains less Trx, GSH, and cysteine, and is compromised in its ability to detoxicate ROS and restore intracellular redox potentials to a reducing range. These differences suggest that more prolonged periods of potential misregulation and sustained oxidative redox potential may occur during thalidomide-induced teratogenesis in the rabbit, disrupting redox-sensitive processes and subsequent downstream events.

NF-κB IS A REDOX SENSITIVE TRANSCRIPTION FACTOR

NF-κB was first identified as a nuclear transcription factor bound to the B-site of the immunoglobulin κ enhancer in B-cells, but it was later determined to have similar binding and gene transactivation activities in many other cell types (6, 7). NF-κB is composed of two subunits, p50 and p65 (RelA), which combine to form a heterodimer in the functional state of mammals such as the rat and rabbit. The p65 (RelA) subunit is regulatory and is bound to an inhibitory protein, I-κB, while sequestered in an inactive state in the cytosol (Fig. 1A). In order for NF-κB to be activated, I-κB is phosphorylated, polyubiquinated, and degraded in the cell's proteosome. The free NF-κB can then translocate into the nucleus, bind to relevant κB enhancer sites in DNA, and initiate gene transcription (27). The p50 subunit accounts largely for the interaction with the DNA binding through a novel DNA-binding motif called a β barrel, consisting of a group of β sheets (15, 30, 60). At the tip of the β barrel is the DNA binding loop that contains a critical cysteine residue, Cys^{62} (35). The importance of Cys^{62} was confirmed by site-directed mutagenesis where Cys^{62} substitution with alanine completely abolished DNA-binding activities (54, 55). Preservation of Cys^{62} in a reduced state is necessary for normal DNA binding to occur (22, 27), which may rely largely on Trx reduction (30, 60). Oxidation of Cys^{62} results in a decreased DNA binding and the subsequent loss of NF-κB-related gene expression (22, 27).

In NF-κB activation/binding studies, Molt-4 cells were exposed to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a glutathione reductase inhibitor that simultaneously increases GSSG and decreases GSH in a dose-dependent manner. Concentrations of 100 μ*M* BCNU caused a 250% increase in GSSG from control while effectively depleting GSH concentrations by 50%, representing an oxidative redox potential shift. Lower BCNU concentrations (3.3–10 μ*M*) increased NF-κB activation fourfold from control. However, upon addition of higher BCNU concentrations (30–100 μ*M*), NF-κB DNA binding decreased by eightfold from control (29). Smaller shifts in redox status, as indicated by relatively low concentrations of GSSG, are apparently not sufficient for NF-κB and I-κB dissociation, but extraordinarily high concentrations of GSSG, indicative of a much greater oxidative redox shift, inhibit NF-κB/DNA binding. It has been proposed that optimal NF-κB activity is achieved when intermediate GSSG concentrations are encountered (29). If intracellular redox

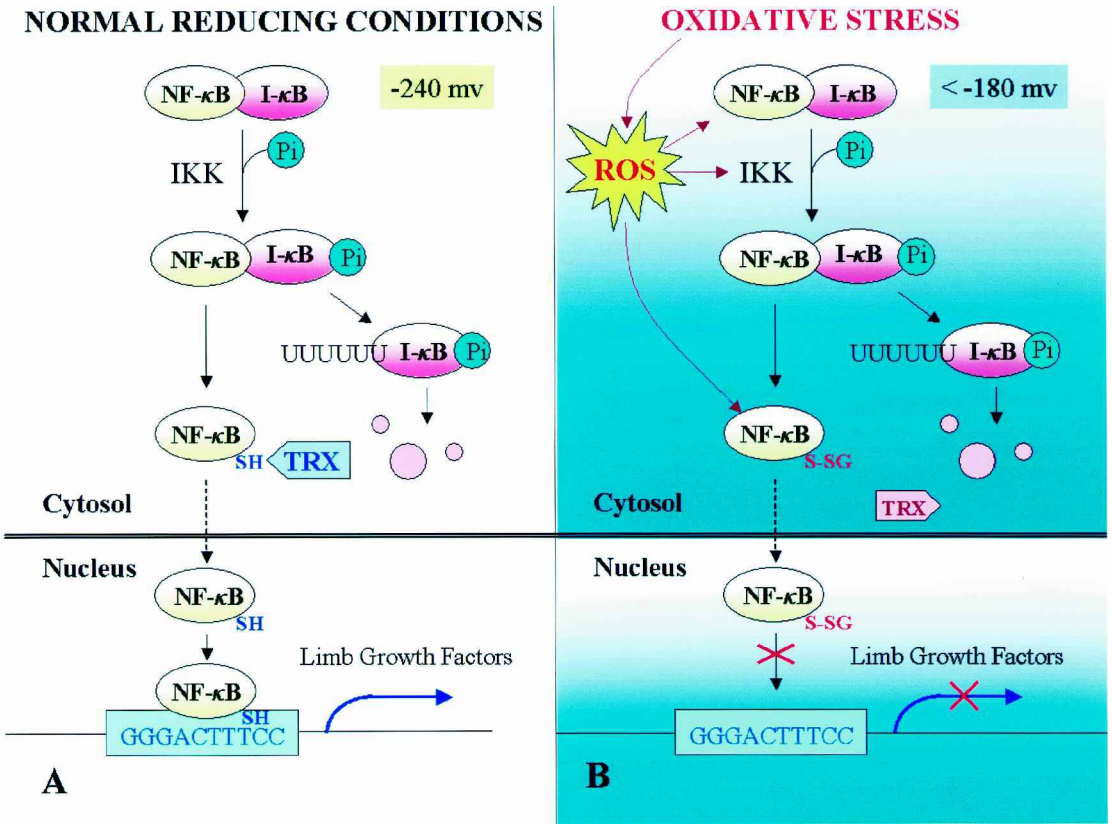


FIG. 1. Activation, translocation, and DNA binding of NF-κB under normal reducing conditions and during oxidative stress. In reducing conditions **(A)**, NF-κB is bound to the inhibitory protein I-κB in an inactive form in the cytosol. Introduction of an activating stimulus, including oxidative stress, initiates the phosphorylation of I-κB by I-κB kinase (IKK), resulting in dissociation of the inhibitory subunit. Subsequent polyubiquitinization (UUU) facilitates proteolysis and destruction of the inhibitory subunit. The free NF-κB, with its reduced sulfhydryl (SH) in the binding domain, is free to translocate to the nucleus where it binds to the κB motif of the promoter and initiates transcription of genes such as the limb growth factors. The enzyme thioredoxin (TRX) is believed to maintain the critical DNA-binding sulfhydryl in its reduced form. In conditions of oxidative stress **(B)**, activation and removal of I-κB and subsequent translocation to the nucleus are likely to occur efficiently, but the critical DNA-binding sulfhydryl is subject to oxidation (*S*-glutathionylation) to NF-κB-S-SG and is unable to bind to the consensus site in the promoter. Low levels of TRX would exacerbate this effect.

status is out of a moderately oxidative range, NF-κB may not be able to functionally mediate signal transduction (Fig. 1B). This distinction makes NF-κB a unique transcription factor in that it reacts differently to fluctuations in redox status as compared with other redox-sensitive transcription factors, such as activator protein-1 (AP-1). As the intracellular environment becomes increasingly oxidative, levels of AP-1 activation and DNA binding increase. In contrast, in an oxidative environment, NF-κB activation increases, but DNA binding decreases (22). NF-κB is located in the cytosol and, following activation, translocates to the nucleus. Therefore, NF-κB is subject to redox microenvironments of both cellular compartments, whereas AP-1 is located in the nucleus and is generally subject to that microenvironment only. As NF-κB can be modulated by either, or both, redox environments, it has a narrower functional range than other redox-sensitive transcription factors.

MOLECULAR MECHANISMS OF LIMB OUTGROWTH INVOLVE REDOX-SENSITIVE REGULATORY PATHWAYS

Proliferation, differentiation, and apoptosis are all essential developmental events that require specific regulation through control of gene expression to evoke the transition from one cellular state to another. Genetic control of developmental processes is largely regulated by the activities of transcription factors. A number of transcription factors and cell signaling elements associated with developmental gene expression have been shown to be subject to redox regulation in other biological systems, but have not yet been systematically evaluated for their role in developing systems. AP-1, p53, NF-κB, and other transcription factors implicated in apoptosis, proliferation, and differentiation are dependent on normal

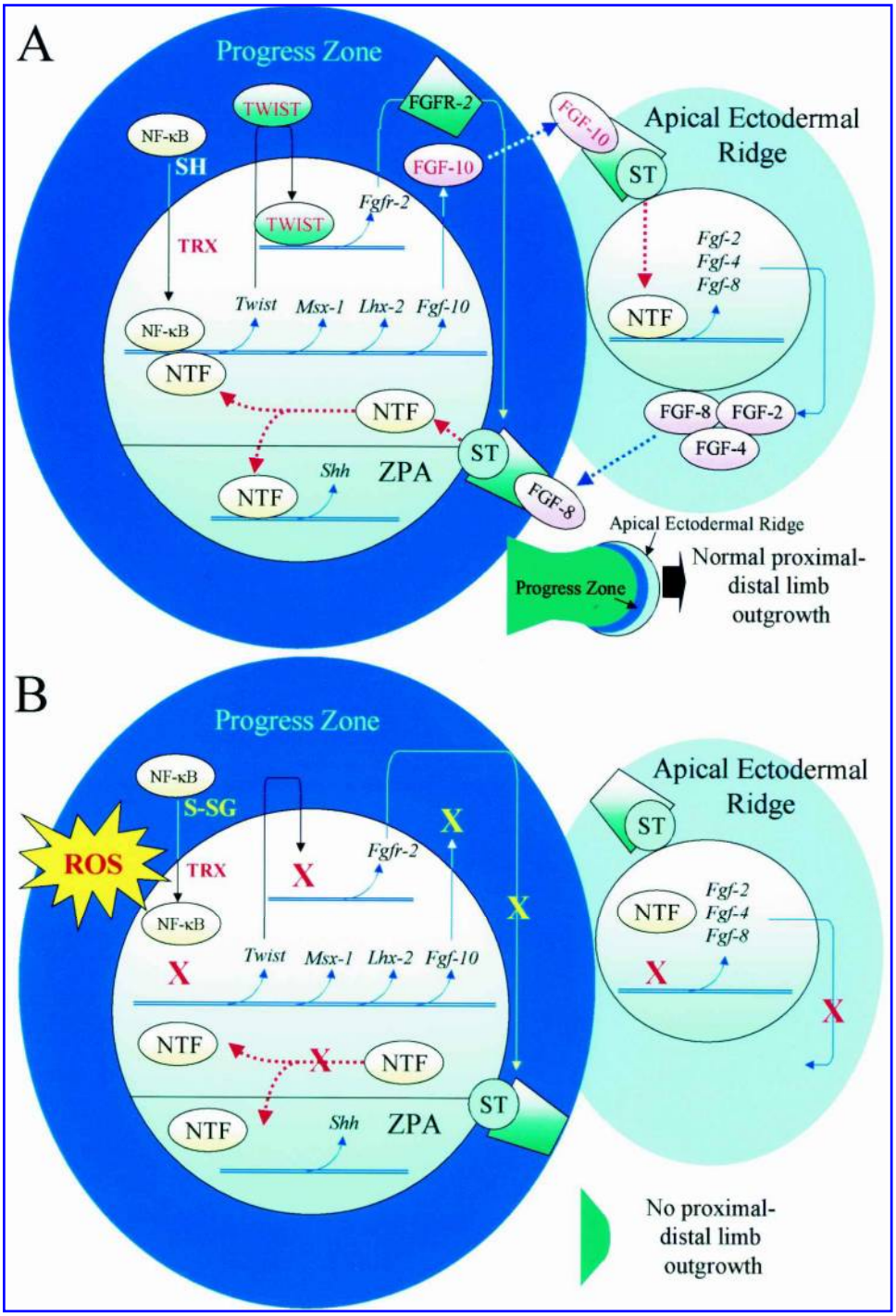


FIG. 2. Proposed model for the NF-κB-mediated redox regulation of limb outgrowth in the PZ and in the AER under normal conditions and during thalidomide-induced oxidative stress (10, 39). Limb outgrowth is maintained by NF-κB-induced expression of growth regulators and transcription factors in the PZ, such as Twist, Msx-1, Lhx-2, and Fgf-10. TWIST is a transcription factor that initiates expression of the FGFR-2 receptor. FGF-10 is a secretable growth factor that diffuses to the AER and initiates expression of additional factors FGF-2, FGF-4, and FGF-8. The latter, in turn, diffuse back to the PZ to activate nuclear transcription factors that maintain expression of critical growth factors. As long as the PZ-AER signaling loop is maintained, limb outgrowth continues unimpeded along the proximal–distal axis. Insults, such as thalidomide exposure, that increase ROS production (as described in Fig. 1) result in the attenuation of NF-kB-mediated gene transactivation, preventing adequate growth factor expression and disturbing the signaling loop required for proximal–distal limb outgrowth. As a result, limb growth is compromised and phocomelia or amelia result.

intracellular redox status for activation and/or DNA binding and subsequent gene transactivation (77, 85).

NF-κB is the *Drosophila* analogue of Dorsal and is known to play a critical specific role in the regulation of limb outgrowth and development (31). Developmental biologists have long understood the essential relationship between the mesoderm of the limb progress zone (PZ) and the apical ectodermal ridge (AER) for productive limb outgrowth (53, 84). Only recently have the underlying molecular signaling pathways been identified and partially characterized. Kanegae and co-workers (43) utilized infection of chick embryos with a nonreplicating adenovirus vector to overexpress two I-κB mutants, I-κBM and I-κBΔUb in attempts to examine the role of NF-κB in the regulation of limb development. I-κBM contains two alanine substitutions for serine-32 and serine-36, the putative sites where I-κB must be initially phosphorylated for NF-κB activation. Because I-κB phosphorylation cannot occur, the NF-κB/I-κB complex does not dissociate and remains sequestered and inactive in the cytosol. The I-κBΔUb mutant does not contain these same serine mutations and is successfully phosphorylated, but the ubiquitination sites have been selectively mutated to lysine residues, preventing ubiquitination and subsequent I-κB degradation. Although NF-κB dissociation from the inhibitory subunit initially occurs, I-κBΔUb reassociates with NF-κB, rendering it inactive in the cytosol before translocation to the nucleus can occur. Limb buds transfected with either I-κB mutant inhibited growth along the proximal–distal axis at the time of infection and were noticeably malformed and reduced in size (43).

Bushdid and co-workers (11) used a slightly different I-κB mutant (I-κBαΔN), which lacks the first 40 amino acids that contain the critical serine residues necessary for I-κB degradation. Much like mutants used in the previous study by Kanegae and co-workers (43), NF-κB is not able to dissociate from I-κB and would remain sequestered in the inactive form in the cytosol. In chick embryos infected prior to the establishment of the limb field, inactive NF-κB was sequestered in the cytosol and produced a 70% incidence of the embryos with morphological limb abnormalities, such as amelia, the absence of limb bud outgrowth, and abnormal AER formation (11). The AER is a group of specialized cells located on the distal portion of the limb bud responsible for proximodistal development through interactions with the underlying mesenchyme. Loss of AER signaling to the underlying mesenchyme via microdissection results in severe stunting of the limb bud (84), illustrating the importance of the AER region during limb development. Both sets of I-κB mutant experiments produced a significant change in expression of mRNA transcripts involved in the limb outgrowth feedback loop [inhibition of msx-1, twist, lhx-2, fgf-10, sonic hedgehog (shh), fgf-8, fgf-2, and derepression of bone morphogenic protein-4 (BMP-4)].

The subset of genes, described above, that operate under control of the redox-sensitive transcription factor NF-κB and were found to be necessary for regulation of limb outgrowth can be organized into the perspective of overall limb development as shown in Fig. 2A. Induction of limb outgrowth along the proximal–distal axis is initiated by growth factors secreted from somitic mesoderm, lateral plate mesoderm, and, likely, the underlying mesonephros. Proximal–distal limb outgrowth is controlled through reciprocal signaling between the tissues of the lateral plate mesoderm and the overlying epithelium. Expression of Fgf-8 is detected early in the presumptive limb field prior to observable morphological changes, and stimulates the proliferation of mesoderm and organizes epithelial cells along the anterior–posterior axis to become the AER (19). As the AER is organized, underlying mesoderm begins to express NF-κB and Fgf-10 and is accompanied by an increase in mesenchymal cell proliferation. Continued expression of Fgf-10 in the distal mesenchyme (PZ) interacts with the AER through an epithelial Fgf receptor isoform (Fgfr2, isoform IIIb) to serve as an "AER maintenance factor" (53). Initiation of Fgf-10 expression is assumed to be due to the activity of the transcription factor NF-κB, although this issue has not been unequivocally resolved. As described in the preceding section, inhibition of NF-κB results in the alteration of expression of several genes in both the PZ and the AER (Fig. 2B). It is not known whether these additional genes are directly responsive to NF-κB or whether their regulation is indirect through NF-κB. Nonetheless, limb outgrowth proceeds when the PZ-AER transcription loop is intact and msx-1, twist, lhx-2, fgf-10, shh, fgf-8, fgf-2, and derepression of BMP-4 all occur in their spatial regions.

Mutations of the *Twist* gene, coding a basic helix–loop–helix transcription factor, have been implicated in the Saethre–Chotzen syndrome in humans where limb defects are part of the observed spectrum of malformations (16, 24, 38, 69, 71). Loss of *Twist* activity seriously compromises limb development because *Twist* is thought to regulate growth through the expression of *Fgfr-1* and *Fgfr-2* (38, 78). *Twist* expression usually occurs uniformly throughout the limb bud mesenchyme, but infection with I-κB mutants produced a marked reduction in the range of normal *Twist* expression. Expression is selectively diminished in the distal mesenchyme of the limb PZ (11, 43). Evidence from I-κB mutant infection studies suggests that NF-κB regulates expression of *Twist* and that it is necessary for normal limb development and outgrowth.

Infection with I-κB mutants also alters *Fgf-8* expression in the limb bud (Fig. 2B). *Fgf-8* expression is normally restricted exclusively to the AER where it is involved in a feedback loop with the underlying mesenchyme (PZ) and the growth factor *Fgf-10* (53, 67) via the interactions with *Fgfr-2b* in the AER and *Fgfr -2c* in the PZ (90). *Fgf-10* is a critical factor during limb development as it contributes to limb bud outgrowth through activation of Fgf-8 expression in the AER. Transgenic mice, deficient in *Fgf-10,* fail to develop any fore- or hindlimb elements and fail to express *Fgf-8* in the AER (44, 57). The *Fgf-10/Fgf-8* feedback loop is what is thought to be primarily responsible for limb bud outgrowth and maintenance of the AER, but not directly involved in limb bud patterning (53). Not only is *Fgf-8* connected to the initiation of limb bud development, but it is also related to the maintenance of the expression of *Shh* in the zone of polarizing activity (ZPA) (88) (Fig. 2). Shh is proposed to be the principle mediator of the polarizing signaling activity along the anterior–posterior axis as it can cause ZPA-like duplications when misexpressed in other portions of the limb bud (14, 72, 88). I-κB mutant infection resulted in the loss of expression of both *Fgf-8* and *Shh* in the AER and ZPA, respec-

tively (43). Interestingly, the expression of genes involved with limb patterning on axes of development, apart from the proximal–distal, such as *cHoxA10*, *cHoxA11*, *cHoxD9*, *cHoxD10*, and *cHoxD13*, were not affected by mutant I-κB retroviral infection (11). These experiments did not investigate the possible misregulation of other AER FGFs, such as *Fgf-2* or *Fgf-4*. *Fgf-2* and *Fgf-4* expression is also necessary for limb bud outgrowth as past experiments have shown that removal of the AER causes stunted growth of the limb bud, but can be rescued with the addition of *Fgf-2*- and *Fgf-4*-soaked beads (64, 84), suggesting some redundancy in limb bud outgrowth pathways. Expression of *BMP-4* was also measured and showed an increase of expression (11). NF-κB has been shown to repress the expression of *BMP-4* in the limb mesenchyme, thereby allowing for limb cell proliferation and outgrowth to occur before the formation of cartilage and limb skeletal elements.

THALIDOMIDE DISRUPTS LIMB OUTGROWTH THROUGH REDOX MISREGULATION OF NF-κB

An accurate hypothesis for the mechanism of thalidomide-induced limb defects must not only explain the molecular relationships between the exposure/environment and the observed effects seen *in vitro*, but must also address species specificity and be consistent with effects produced in the whole animal. The multicompartment redox regulation of NF-κB, as described above, would imply that both sensitive and resistant species would activate NF-κB and facilitate its translocation into the nucleus within the PZ, but that DNA binding within the nucleus would be inhibited or attenuated only in the sensitive species.

Misregulation of NF-κB activity alters the expression of critical developmental genes in each regulatory region of the limb bud, *Twist* and *Fgf-10* in the PZ mesenchyme, *Fgf-8* in the AER, and *Shh* in the ZPA (11, 43). As NF-κB acts either directly or indirectly to control the expression of limb bud development genes in these areas, it is a prime target for redox misregulation in thalidomide-induced limb teratogenesis. Thalidomide produces oxidative stress (32, 34, 68, 74), and as the redox potential of the cell shifts to a more oxidative environment, NF-κB becomes more easily activated. Studies by Hansen *et al.* (33) utilizing transient transfection of a green fluorescent protein (GFP) reporter vector suggest that NF-κB in the PZ of both rat and rabbit limb buds may be constitutively active, due to the very positive redox potentials found in these regions. Using DCF to localize the region of highest ROS accumulation in the cell, thalidomide exposure in rabbit LBCs caused the greatest area of fluorescence in the nucleus, whereas thalidomide-treated rat LBCs showed no such localization (34). These findings correlated with 5-chloromethylfluorescein diacetate (CMFDA) staining for reduced GSH in rat and rabbit LBCs (Fig. 3). GSH was depleted in the cytosol, but nuclear concentrations were unaffected. Conversely, rabbit LBCs, which do not contain as robust cytosolic GSH concentrations as the rat, showed depletion of both cytosolic and nuclear GSH with similar thalidomide treatments (34).

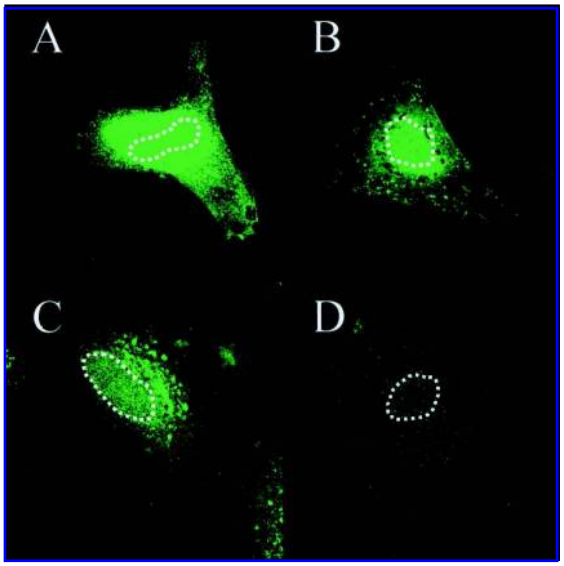


FIG. 3. LBC stained with CMFDA for GSH localization. Rat LBCs treated without **(A)** and with **(B)** 100 μ*M* thalidomide and rabbit LBCs treated without **(C)** and with **(D)** 100 μ*M* thalidomide for 120 min are presented. Nuclei are outlined with a white dotted line. Cells were viewed by confocal microscopy. Thalidomide treatments in rat LBCs deplete cytosolic GSH, but nuclear GSH remains. In rabbit LBCs, untreated cells have less cytosolic GSH. Thalidomide treatment causes a complete loss of GSH in both the cytosol and nucleus.

The preferential increase of nuclear ROS and loss of GSH suggest that NF-κB misregulation most likely occurs in the nuclear compartment rather than the cytosol and transpires at the DNA binding level. Evaluation of NF-κB/DNA binding efficiency following thalidomide exposure was determined in both rat and rabbit LBCs using a GFP reporter vector. Thalidomide caused a substantial decrease in GFP production in rabbit LBCs, but rat LBCs were unaffected (33), indicating rabbit susceptibility to thalidomide-induced misregulation of NF-κB binding activity. DNA binding and subsequent GFP production could be restored to control levels with cotreatments of PBN or *N*-acetylcysteine and indicate that thalidomide-induced NF-κB/DNA binding misregulation is mediated by free radical formation or modulation of intracellular redox potential (33).

Many of the preliminary studies implicating thalidomide and oxidative stress in limb reduction defects have been performed *in vitro*. However, estimation of thalidomide effects *in vivo* is more difficult and offers many unique challenges. It is very difficult to directly assess thalidomide-induced misregulation of NF-κB binding as was demonstrated *in vitro*. To best estimate the effects of thalidomide on NF-κB activities *in vivo*, whole mount *in situ* hybridizations were performed on genes that had previously been shown to be down-regulated in the limb as a consequence of NF-κB misregulation (11, 43), namely *Twist, Shh, Fgf-10,* and *Fgf-8* in both rat and rabbit embryos treated *in utero* (33).

Twist was shown to be unaffected with thalidomide treatment in the gestational day (GD) 11 and 13 rat embryos and showed expression in the mesenchyme underlying the AER, or the PZ

(Fig. 4). Following thalidomide treatment, rabbits of similar developmental age (GD 10–12) showed a complete loss of *Twist* expression early in limb development (GD 10), but expression was slowly restored in older embryonic limbs (GD 11–12) and never reached levels comparable to those in untreated control rabbit embryos (Fig. 5) (33). Although the loss of *Twist* expression would contribute to the disruption of the Fgf-8/Fgf-10 feedback loop and limb dysmorphogenesis, the effect of thalidomide on both *Fgf-8* and *Fgf-10* expression is equally as profound. *Fgf-8* expression in the AER is unaffected by thalidomide exposure in rat embryonic limbs (GD 11 and 13) (Fig. 4).

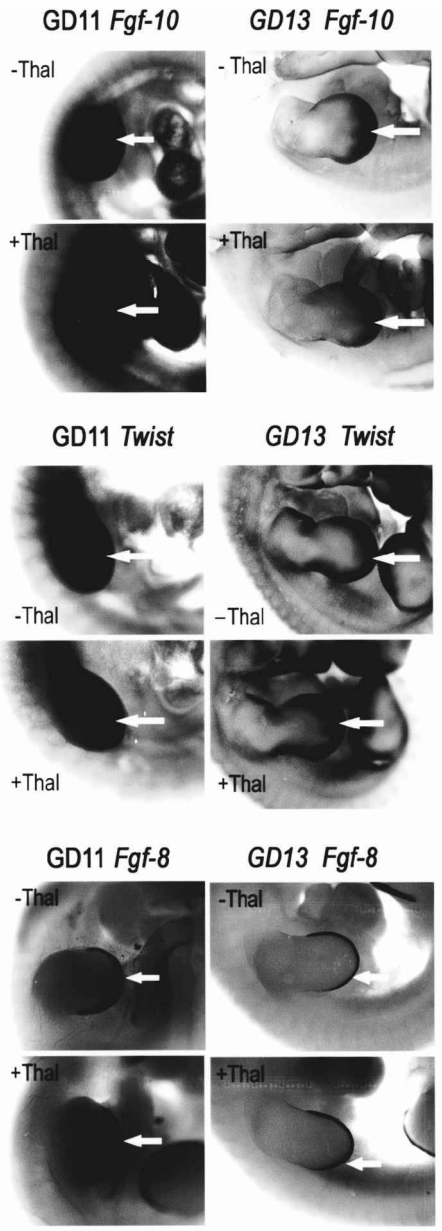


FIG. 4. ***In situ*** **hybridization of GD 11 and GD 13 rat embryos treated with thalidomide.** White arrows point to regions of expression of *Fgf-10*, *Twist*, and *Fgf-8*. Thalidomide does not affect the expression of limb bud outgrowth gene expression.

However, *Fgf-8* expression in the rabbit AER is undetectable with thalidomide treatment during the early stages of limb initiation and development (GD 10), but increases as limb development progresses (GD 11–12). *Fgf-8* expression is localized to the AER, but the restoration is seen only in limited regions of the AER, not uniformly throughout the entire AER (Fig. 5) (33). Recent studies where *Shh* was deleted in transgenic mice showed that the loss of *Shh* expression also decreased the expression of *Fgf-8*, but the reduction of *Fgf-8* expression only occurred in the posterior portion of the limb, the area overlying the ZPA, whereas the remainder of the AER was unaffected (49). These same manifestations were evident in rabbit embryos treated with thalidomide and, although not directly studied, suggest that Shh is also affected (Fig. 2).

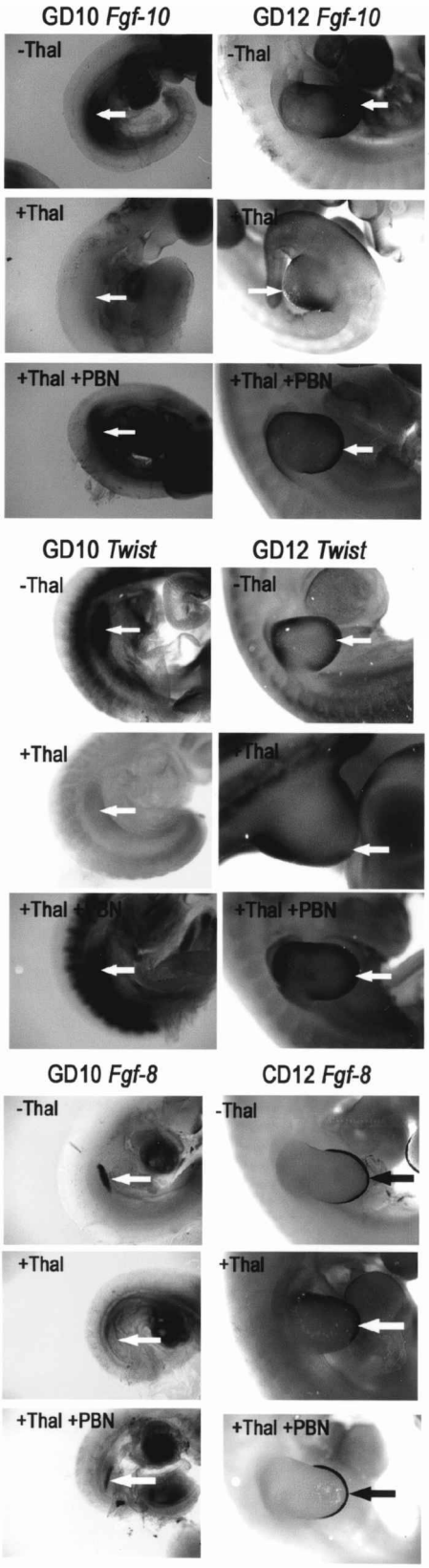
Finally, *Fgf-10* expression was evaluated in the PZ. True to form, rat embryos treated with thalidomide did not show any significant decrease in *Fgf-10* expression on either GD 11 or 13 (Fig. 4). Rabbit embryos treated with thalidomide showed very little *Fgf-10* expression on GD 10, but increased on subsequent GDs. Interestingly, expression in treated rabbit embryos is still very low at the very distal tip of the PZ, implicating this region as a possible target for misregulation and area of highest oxidative stress (Fig. 5) (33). Supporting this idea, rat and rabbit limb buds treated with thalidomide *in utero* were stained with mercury orange to determine the GSH distribution. Although both rat and rabbit showed high concentrations of GSH in the overlying AER with or without thalidomide treatment, only the underlying PZ of the rabbit was depleted of mercury orange staining, indicating that the mesenchyme of the PZ is the region most affected by thalidomide-induced oxidative stress (33).

The utility of implicating thalidomide-induced ROS production in misregulation of limb outgrowth rests on the assumption that elimination of ROS would also restore normal signaling. As Parman and Wells (68) showed in the protection against DNA oxidation, the use of a free radical spin trap should reverse the deleterious effects of thalidomide in the whole animal, including the misregulation of gene expression in the rabbit as described above (33). Cotreatment of pregnant rabbits with PBN (Fig. 5) did, in fact, prevent the loss of transcriptional activity caused by thalidomide. This provides a compelling argument in support of the hypothesis that thalidomide selectively induces the production of ROS, resulting in loss in DNA binding of NF-κB, reduction of gene expression, and attenuation of limb outgrowth.

Although rabbit embryos treated with thalidomide showed a temporary decrease of *Fgf-10* expression (GD 10), restoration of expression later in development corresponds to presented terata. Rabbit embryos rarely exhibit thalidomide-induced amelia, but rather the primary limb reduction defect is phocomelia. As the loss of *Fgf-10* expression is transient, limb bud development and outgrowth may simply be retarded rather than inhibited and would yield limb reduction defects such as phocomelia, not amelia.

PERSPECTIVES

The data presented in this editorial support the hypothesis that thalidomide-induced limb defects involve ROS-mediated



inhibition/attenuation of redox-sensitive transcription factors in sensitive species. The fundamental mechanism by which these effects are realized is believed to be the formation of a GSH-protein mixed disulfide (*S*-glutathionylation) in the critical DNA-binding domain (Cys^{62}) of the p50 subunit of NF-κB that prevents efficacious binding and gene transactivation. *S*-Glutathionylation of cellular protein occurs extensively when concentrations of GSSG increase during oxidative stress, and the consequences are known to affect several important cell functions (18, 48). This process has attracted considerable recent attention due to demonstrations of important regulatory roles in other systems (18, 47, 48). Pineda-Molina *et al.* (70) suggest a complex scenario of regulation through response to changes in the redox environment that could involve several different modifications at the critical DNA-binding cysteine in the transcription factor NF-κB that could impart selective binding and reactivation characteristics. Evidence is provided to support *S*-glutathionylation (-S-SG) and sulfenic acid (-SOH) formation in the reversible inhibition of the transcription factor NF-κB when GSH/GSSG ratios are shifted to produce a more oxidizing redox environment. *In vitro* experiments showed that GSH and GSSG concentrations of 1.0 and 0.1 mol of [^{3}H] GSH/mg of protein, respectively, result in a 40–70% inhibition of the binding of the p50 wild-type subunit. Further reduction of the GSH/GSSG ratios to 0.4 (GSH) and 0.1 mol (GSSG) of [^{3}H]-GSH/mg of protein caused complete inhibition of DNA binding. Depending on the magnitude of change in the intracellular redox potentials and intrinsic cellular response capacities, a broad spectrum of covalent modifications on the DNA-binding cysteine may occur in NF-κB, ranging from reversible *S*-cysteinylation (-*S*-cys), *S*-glutathionylation (-S-SG), sulfenic acid formation (-SOH), and *S*-nitrosoglutathionylation (GSNO) to the further extreme of irreversible oxidative modifications such as sulfinic acid ($-SO_2H$) and sulfonic acid ($-SO_3H$). Reversibility of inhibition and restoration of DNA binding are largely dependent on the availability of sufficient GSH concentrations (reducing conditions) and enzymes, such as Trx and other protein disulfide reductases, which can be regulated in a cell-specific manner (23, 41, 47, 48). We propose that heterogeneity in the redox-inhibition states of NF-κB may be cell- and tissue-specific and regulated, in part, by selective abilities to respond to chemical and environmental changes. An identical external stimulus may induce different changes in intracellular redox status in order to stimulate proliferation in one cell type through reversible inhibition and differentiation or death in another due to the formation of irreversible thiol modifications. This phenomenon may have particular relevance to broader mechanisms in embryonic development and stem cell biology where rapid changes in cell

FIG. 5. ***In situ*** **hybridization of GD 10 and GD 12 rabbit embryos treated with thalidomide and co-treated with both thalidomide and PBN.** White arrows show regions of expected gene expression. Rabbit embryos treated with thalidomide showed a substantial decrease of *Fgf-10*, *Twist*, and *Fgf-8* expression in specific regions of the developing limb. Cotreatment with PBN blocked the deleterious effects of thalidomide and restored normal expression patterns.

function, related to altered patterns of gene expression, are often observed in the absence of any other obvious signals.

Although we have focused on NF-κB as the critical transcription factor involved in the misregulation of limb outgrowth, we do not imply that other redox-sensitive transcription factors and signal transduction elements are not also significantly affected by variations in redox status. *S*-Glutathionylation of the activator protein-1 (AP-1, c-jun) complex has been shown to affect DNA binding in a manner similar to that described for NF-κB (48). This system may not be directly involved in the cascade that regulates limb outgrowth, but it may have particular relevance in the maintenance and recovery of intracellular redox status following oxidative insult. A good example is the AP-1-mediated induction of the glutamate-cysteine ligase that is rate limiting for *de novo* GSH synthesis and responsible for increasing GSH concentrations (86). Collectively, a number of redox-sensitive transcription factors and signal transduction proteins (NF-κB, AP-1, p53, SP-1, mitogen-activated protein kinase, c-Jun N-terminal kinase, etc.) may be responsible for sensing the redox-related changes elicited through environmental and chemical insult to result in the inducible expression of genes responsible for controlling important developmental processes, such as proliferation, differentiation, and apoptosis (2, 18, 39, 59, 77). This represents a novel level of gene regulation during embryogenesis. "Environmental factors" that contribute to cell signaling in early development could be mediated through ROS and redox regulation in a manner that has not been previously considered. Decreased gene expression in the developing limb due to the inhibition or attenuation of redox-sensitive transcription factors provides a superior rationale for understanding mechanisms of other known teratogens, which also increase the production of ROS. Due to the dynamic nature of oxidative stress and the individual cell's dependence on pathways to restore normal reducing conditions, cell and species sensitivity to teratogens may be based on their antioxidant and response status. The intrinsic ability of a cell or tissue to restore reducing conditions following an oxidative insult may determine whether misregulation of redox-sensitive transcription factors occurs and promotes dysmorphogenesis.

Thalidomide elicits a more positive, oxidized intracellular redox potential, resulting in the misregulation of NF-κB. Diminished NF-κB binding to DNA in the limb PZ leads to loss or reduction of gene expression (*Twist*, *Fgf-10, Fgf-8*, and *Shh*), which is critical for the continued proliferation and differentiation during limb development, whereas other patterning genes, such as the Hox genes, remain unaffected. These findings account for thalidomide's effect on limb reduction while still maintaining normal patterning of the fingers and hand, mimicking the most common human thalidomide-induced defect, phocomelia (76).

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ABBREVIATIONS

AER, apical ectodermal ridge; AP-1, activator protein-1; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BMP-4, bone morphogenic protein-4; CMFDA, 5-chloromethylfluorescein diacetate; DCF, dichlorofluorescein; Fgf-2, fibroblast growth factor-2; Fgf-4, fibroblast growth factor-4; Fgf-8, fibroblast growth factor-8; Fgf-10, fibroblast growth factor-10; Fgfr-1, fibroblast growth factor receptor-1; Fgfr-2, fibroblast growth factor receptor-2; GD, gestational day; GFP, green fluorescent protein; GSH, reduced glutathione; GSSG, glutathione disulfide; Igf, insulin growth factor; LBC, limb bud cell; NF-κB, nuclear factor-κB; PBN, α-phenyl-*N*-*tert*-butylnitrone; PZ, progress zone; ROS, reactive oxygen species; Shh, sonic hedgehog; Trx, thioredoxin; ZPA, zone of polarizing activity.

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Address reprint requests to:
Craig Harris, Ph.D.
Toxicology Program
Department of Environmental Health Sciences
University of Michigan
1420 Washington Heights
Ann Arbor, MI 48109-2029

E-mail: charris@umich.edu

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