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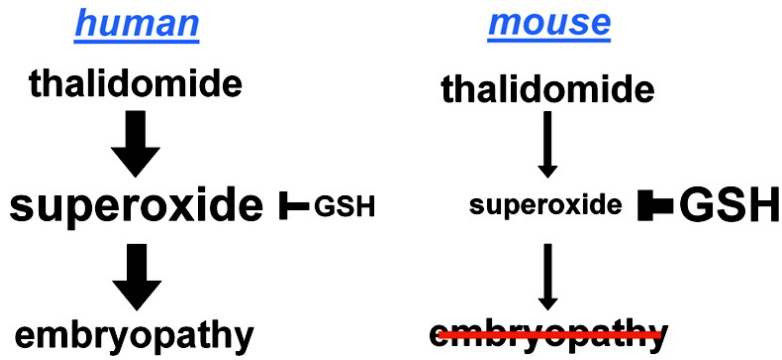

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Thalidomide Resistance Is Based on the Capacity of the Glutathione-Dependent Antioxidant Defense

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Abstract: Thalidomide as an effective treatment for multiple myeloma and leprosy has also caused birth defects in thousands of children five decades ago particularly in Europe. Thus its use in humans remains limited. The rapid and fatal approval of thalidomide at that time ultimately was a consequence of the sole use of thalidomide-insensitive species in animal toxicity tests. Here, we aimed at elucidating the molecular basis for the resistance of mice to thalidomide teratogenicity. By using hydroethidine staining we demonstrate that thalidomide induces the formation of superoxide in embryonic fibroblasts of thalidomide-sensitive species but not in those of mice. As determined by trypan blue staining, scavenging of superoxide prevents thalidomide-induced apoptosis, a marker for thalidomide teratogenicity. Mouse embryonic fibroblasts are found to have higher glutathione levels than those of sensitive species and can be sensitized for thalidomide by glutathione depletion with diethyl maleate or diamide. Accordingly, experimental increase of glutathione levels in human embryonic fibroblasts by adding *N*-acetyl cysteine or glutathione ethyl ester to the culture medium counteracts thalidomide-induced apoptosis. Finally, we show that thalidomide-induced molecular pathology downstream of superoxide is essentially identical in human and sensitized mouse embryonic fibroblasts. In conclusion, thalidomide-resistance is based on the capacity of the glutathione-dependent antioxidant defense. We provide a basis to pharmacologically overcome the limitations of thalidomide use at humans and describe substantial differences between human and mouse embryonic cells regarding the protection against oxidative stress.

Keywords: thalidomide; mouse embryonic cells; drug resistance; teratogenic; superoxide; glutathione; antioxidant defense; apoptosis

Introduction

Administered to pregnant women as a sedative in the late 1950s, thalidomide [2-(2,6-dioxopiperidin-3-yl) isoindoline-1,3-dione] was found to be responsible for a broad spectrum of birth defects, most notably limb truncations, and was subsequently removed from the market.[1] Because of its anti-inflammatory and antiangiogenic properties thalidomide has regained new interest and is currently used as a treatment for several diseases, including multiple myeloma and leprosy.[2] Nevertheless, its teratogenic effects limit the use of thalidomide in humans. To overcome this limitation it is necessary to elucidate the thalidomide-induced molecular pathology during embryogenesis causing embryopathy.

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Thalidomide teratogenicity is species-specific. Since mice and rats are insensitive,[3] we have previously established the chicken embryo as a thalidomide-sensitive model system and were able to demonstrate that thalidomide-induced limb truncations are the consequence of a massive up-regulation of apoptosis during embryonic limb development.[4] Thalidomide induces apoptosis in primary embryonic fibroblasts of chicks (CEFs) and humans (HEFs) suggesting embryonic fibroblasts as suitable in vitro models.[4] Using CEFs, HEFs and the chicken embryo we have found that thalidomide up-regulates the expression of bone morphogenetic proteins (Bmps) with two major consequences: first, hyperexpression of the Bmp target gene and secreted Wnt antagonist Dickkopf1 (Dkk1), and second, enhanced activity of the phosphatase PTEN with subsequent suppression of the PI3K/Akt pathway. Both events result in increased Gsk3β activity leading to a down-regulation of β-catenin-mediated transcriptional activity. Suppression of both Wnt and Akt signaling finally leads to caspase-dependent apoptosis mediated by the intrinsic mitochondrial and the Fas death receptor pathway.[4,5] In embryonic fibroblasts of mice (MEFs), thalidomide neither up-regulates Bmp or Dkk1 expression nor induces apoptosis.[4] Thus, embryonic fibroblasts seem to be suitable for investigating the species specificity of thalidomide teratogenicity.

Using New Zealand White rabbits, another thalidomide-sensitive species, the generation of oxidative stress has been demonstrated as an essential prerequisite for thalidomide teratogenicity.[6] For HEFs we have shown that thalidomide-induced Bmp expression is mediated by yet unidentified reactive oxygen species (ROS).[4] ROS, including radical (e.g., superoxide anion radical and hydroxyl radical) as well as nonradical species (such as peroxides), may be generated in cells exposed to various xenobiotics that directly or indirectly interact with molecular oxygen, e.g. resulting in the one-electron reduction of O_2 to superoxide. Further reduction yields hydrogen peroxide and the hydroxyl radical. Oxidative stress is the result of an excessive ROS generation overwhelming the intracellular antioxidant defense. Under these conditions ROS induce apoptosis by activating death-signaling pathways. The antioxidant defense includes superoxide dismutases (SOD), catalyzing the dismutation of superoxide to hydrogen peroxide and oxygen, and enzymes that reduce hydrogen peroxide to water, either by means of a dismutation (catalases, CAT), or at the expense of glutathione (GSH) (GSH peroxidases). In addition to its important role as a cosubstrate for GSH peroxidases, GSH protects cells from oxidative stress induced by xenobiotics by direct inactivation through GSH-S-transferase (GST)-mediated coupling of GSH to electrophilic xenobiotics and by direct nonenzymatic scavenging of ROS.[7,8]

Here we demonstrate that superoxide is a major ROS mediating thalidomide-induced apoptosis and that GSH depletion enhances cellular responsiveness to thalidomide. The establishment of mammalian in vivo models is a prerequisite to study the mechanisms of thalidomide teratogenicity with highest relevance for humans. We provide a basis for developing thalidomide-sensitive mice by describing means of sensitizing MEFs for thalidomide and by unraveling the molecular basis of thalidomide-resistance.

Experimental Section

Cell Culture and Reagents. Isolation of CEFs, MEFs and HEFs and preincubation before drug treatment was done as described.[4] Treatment with racemic thalidomide (38.7 μM; Sigma, St. Louis, MO), juglone (Sigma), mouse Noggin (300 ng/mL; R&D Systems), antihuman-DKK1 antibody (350 ng/mL; R&D Systems), bpV(pic) (80 nM; Alexis), Gsk3-inhibitor IX (BIO; 500 nM; Calbiochem, San Diego, CA), phenyl *N*-*t*-butylnitrone (PBN; 2 mM; Sigma), polyethylene glycol (PEG)-coupled SOD, PEG-CAT, MnTBAP, NAC or GSH ethyl ester (all from Sigma) was performed in Opti-MEM1 (Invitrogen, Carlsbad, CA) plus 1% FCS. Unless otherwise noted, all treatments were done for six hours. Cells were pretreated with inhibitors, diethylmaleate (DEM), diamide (both from Sigma) or PEG-coupled enzymes for 30 min before addition of thalidomide, juglone or DMSO (solvent control). For DEM or diamide treatment embryonic fibroblasts in passages 4−6 with 75−85% density were used.

Detection of Superoxide. Cells were cultured with thalidomide or juglone for one hour as described.[4] Following washing with PBS, cells were incubated with 2 μM hydroethidine (HE; Sigma) for 30 min at 37 °C. After two further washes cellular oxidation of HE was detected by fluorescence microscopy (excitation, 490 nm; emission, 590 nm).

Fertilized eggs were purchased from Deindl GmbH (Rietberg-Varensell, Germany). Embryos were incubated, staged according to Hamburger and Hamilton (HH) and exposed to thalidomide (Sigma) at 0.75 mg/kg egg weight at HH stages 4/5 as described before.[4] Superoxide in dissected limb buds was detected by hydroethidine staining according to Schnabel et al.[9]

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Determination of Glutathione Content. Total glutathione content was determined enzymatically according to Anderson[10] with minor modifications. Briefly, cells were lysed by scraping them in ice-cold HCl (10 mM) followed by freeze/thaw lysis of cells and centrifugation to remove cell debris. Aliquots of the supernatants were kept for protein determination according to Bradford. For glutathione determination, protein was precipitated from the supernatant with 5% (w/v, final concentration) 5-sulfosalicyclic acid on ice. Samples were vortexed and centrifuged and total glutathione (GSH plus GSSG) was determined from the supernatants using 5,5′-dithionitrobenzoic acid in the presence of NADPH and glutathione reductase.

RT-PCR, Detection of Cell Death and Statistical Analyses. RNA isolation and semiquantitative RT-PCR were performed according to standard protocols. Primer sequences and PCR conditions are available upon request. Cell death in primary embryonic fibroblasts was measured by trypan blue staining as described.[4] Densitometry was performed with ScionImage software. Two-tailed Student's *t* tests were done using Microsoft Excel.

Results

Thalidomide Induces the Generation of Superoxide. CEFs, MEFs and HEFs were incubated with thalidomide for one hour and stained with hydroethidine to detect superoxide. Thalidomide induced the generation of superoxide in primary embryonic cells of thalidomide-sensitive species (CEFs, HEFs) but not in those of thalidomide-insensitive mice (MEFs) (Figure 1A). Juglone, a redox-cycling agent and potent inducer of ROS generation, caused the formation of superoxide during a one hour incubation in all three cell types, but least so in MEFs (Figure 1A).

The application of thalidomide at 750 μg/kg egg weight to HH stages 4/5 chicken embryos causes apoptosis in developing limb buds at HH stages 23/24 resulting in limb truncations.[4] Using these conditions, we detected superoxide in 54% (15 of 28) of the individual limb buds of thalidomide-exposed HH stages 21/22 embryos with no difference between wing and hindlimb buds (Figure 1B). We did not detect superoxide in limb buds of thalidomide-treated embryos at HH stages 23−26 or in limb buds of solvent-treated control embryos (data not shown).

Scavenging of Superoxide Prevents Thalidomide-Induced Apoptosis. Thalidomide-induced apoptosis at 38.7 μM was about 4.5 to 7.5-fold above controls after six hours of incubation in CEFs and HEFs (Figure 2A−D). Depletion of GSH with diethyl maleate (DEM), a well-known GST substrate, clearly increased thalidomide-induced apoptosis (Figure 2A). The SOD mimetic MnTBAP completely abrogated thalidomide-induced apoptosis in CEFs and HEFs at 150 μM (Figure 2B,C). PEG-coupled (cell-permeant) SOD completely abolished thalidomide-induced apoptosis in a concentration-dependent manner (Figure 2D). Moreover, thalidomide-induced apoptosis was significantly attenuated by about 30% by PEG-coupled CAT (Figure 2D). These data demonstrate that thalidomide-induced apoptosis is mainly mediated by superoxide and, to some extent, also by its dismutation product, hydrogen peroxide.

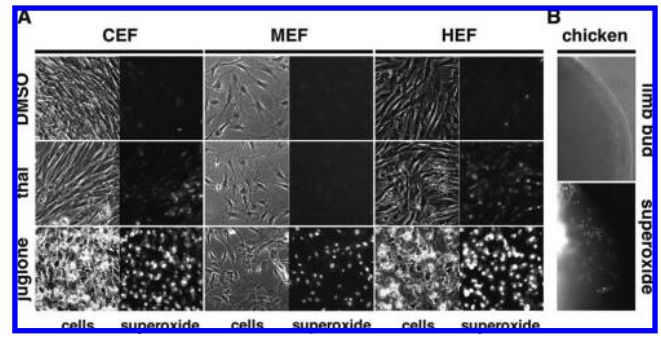

Figure 1. Thalidomide induces superoxide formation in CEFs, HEFs and in limb buds. (A) Embryonic fibroblasts were incubated with 38.7 μM thalidomide, 12.5 μM juglone or DMSO (solvent control) for one hour, followed by labeling with hydroethidine for visualization of superoxide formation. The cells are shown as phase contrast (left column) and fluorescence images (right column), with fluorescence resulting from hydroethidine oxidation products derived from interaction with superoxide. (B) Wing bud (anterior part) of a thalidomide-treated HH stage 20/21 embryo. Superoxide formation is indicated by the bright fluorescent spots. Phase contrast (top) and fluorescence (bottom) images; thal, thalidomide.

Supernatants of Thalidomide-Treated CEFs Induce Apoptosis in MEFs. In order to unravel the molecular basis of thalidomide-resistance of mouse embryonic cells, we initially investigated whether supernatants of thalidomide-treated CEFs may induce apoptosis in CEFs and MEFs. Exposure to thalidomide resulted in an about 5.5-fold enhanced apoptosis in CEFs but not in MEFs after 6 h of incubation (Figure 3A,B). Following 1 h of preincubation with serum-reduced medium, we treated CEFs and MEFs (hereafter referred to as "acceptor CEFs/MEFs", aCEFs/aMEFs) for 6 h with the supernatant of other CEFs (hereafter referred to as "donor CEFs", dCEFs), which had been exposed to thalidomide or solvent (as a reference) for different periods of time. The 1.5 h supernatant of thalidomide-treated dCEFs induced apoptosis in aCEFs but not in aMEFs (Figure 3B), and this effect was less pronounced than that elicited by the direct application of thalidomide (= 0 h supernatant; Figure 3A,B). The free radical trapping agent PBN, Dkk1 blocking antibodies (αDkk1) and inhibitors of Bmps (Noggin), PTEN (dipotassium bisperoxo (pyridine-2-carboxyl) oxovanadate, [bpV(pic)]) or Gsk3β (BIO) significantly attenuated apoptosis induced by direct application of thalidomide (Figure 3A) or by the 1.5 h supernatant (data not shown). The 3 h supernatant of thalidomide-treated dCEFs did not cause apoptosis in aCEFs or aMEFs (Figure 3B), implying that neither thalidomide nor apoptosis-inducing metabolites were present in the supernatant after 3 h of

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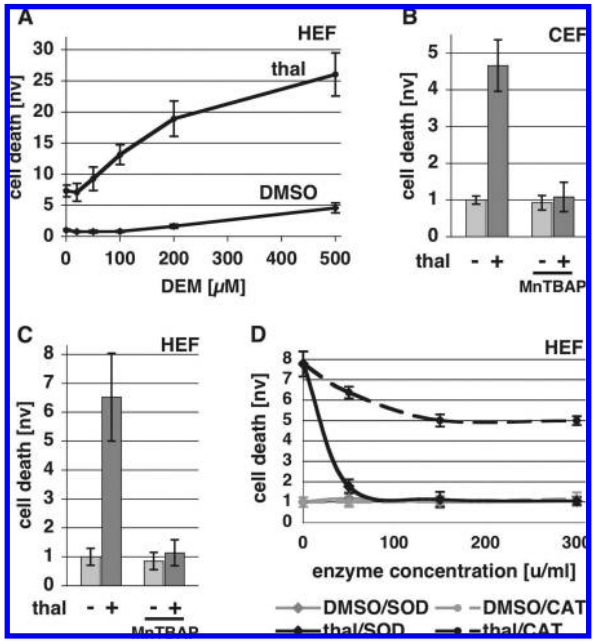

Figure 2. Thalidomide causes the generation of superoxide to induce apoptosis in CEFs and HEFs. HEFs (A, C, D) or CEFs (B) were preincubated with DEM (A), PEG-coupled SOD or CAT (D) at the indicated concentrations or with 150 μM MnTBAP (B, C) before addition of thalidomide (+, thal) or a solvent control (−, DMSO). After six hours of incubation the ratios of dead to live cells were determined and normalized to the negative controls (solvent-treated cells). A value of 1 corresponds to 1−2% cell death. The data represent the mean ± SD from six (B, C) or eight (A, D) individual experiments; nv, normalized values.

incubation, which was expected since the drug rapidly degrades in aqueous solutions. Both the 5.5 h and the 7.5 h but not the 16 h supernatant of thalidomide-treated dCEFs induced apoptosis in aCEFs to a similar extent as a direct thalidomide treatment (Figure 3B). This might be explained by the thalidomide-induced secretion of Bmps, Dkk1 or other pro-apoptotic proteins. In line with this hypothesis, these effects were significantly reduced by Noggin or αDkk1 and by inhibitors of downstream molecules, bpV(pic) or BIO, but not by PBN, which neutralizes ROS that are suggested to act upstream of Bmp and Dkk1 in thalidomide-induced molecular pathology (Figure 3C). Importantly, these supernatants also caused apoptosis in aMEFs (Figure 3B,D), and these effects were also reduced by Noggin, αDkk1, bpV(pic) or BIO, but not by PBN (Figure 3D, data not shown). In summary, these data suggest that the molecular basis for thalidomide resistance of MEFs is upstream of Bmp signaling.

Impairment of the Antioxidant Defense Sensitizes MEFs to Thalidomide. At low concentrations DEM depletes GSH without affecting cell viability but at higher concentrations the GSH level is reduced to such an extent that the cell undergoes apoptosis.[11] Dose−response experiments showed that DEM induced apoptosis at lower concentrations in HEFs compared to MEFs (Figure 4A). Individual treatment with DEM or diamide, another GSH depletion reagent that, in contrast to DEM, is independent of intracellular GST activity, did not induce apoptosis in MEFs at concentrations up to 300 or 25 μM, respectively. However, both DEM at 200 and 300 μM or diamide at 10 and 25 μM enabled thalidomide to induce apoptosis in MEFs (Figure 4B,C). Furthermore, we found that MEFs have an about 5-fold higher glutathione level than thalidomide-sensitive embryonic fibroblasts (CEFs, HEFs) and that treatment with 300 μM DEM lowers the glutathione level of MEFs below that of CEFs and HEFs (Figure 4D). In line with the hypothesis that the intracellular GSH content governs thalidomide sensitivity or resistance, increasing GSH levels in HEFs with N-acetyl-cysteine or GSH ethyl ester clearly reduced thalidomide-induced apoptosis (Figure 4E,F).

Thalidomide-induced apoptosis in embryonic cells of sensitive species is a consequence of enhanced Dkk1 expression.[4] In MEFs, a single treatment with thalidomide for six hours did not enhance Dkk1 expression as determined by semiquantitative RT-PCR. In contrast, DEM at 300 μM induced a slight Dkk1 up-regulation (Figure 5A). This was expected since incubation with DEM is envisaged to lower GSH levels to an extent that results in an impaired reduction of hydrogen peroxide by GSH peroxidases, causing the elevation of hydrogen peroxide steady-state levels. Hydrogen peroxide, in turn, has been shown to induce Dkk1 expression in MEFs.[12,13] However, thalidomide potentiated DEM-induced Dkk1 expression (Figure 5A). Importantly, thalidomide-induced apoptosis in DEM-pretreated MEFs was reduced by PBN or MnTBAP as well as by Noggin, Dkk1 blocking antibodies (αDkk1), bpV(pic) or BIO (Figure 5B,C).

Juglone already at 100 nM induced the generation of superoxide (data not shown) but not apoptosis in MEFs (Figure 5D). In combination with 100 nM juglone, thalidomide induced apoptosis in MEFs about 4-fold above controls, and this effect was neutralized by BIO (Figure 5D). Summarized, our data demonstrate that MEFs harbor a more efficient antioxidant defense against thalidomide-induced ROS than CEFs and HEFs. This strongly suggests that thalidomide insensitivity of MEFs is based upon a higher antioxidant capacity, in particular on higher glutathione levels, compared to thalidomide-sensitive embryonic fibroblasts.

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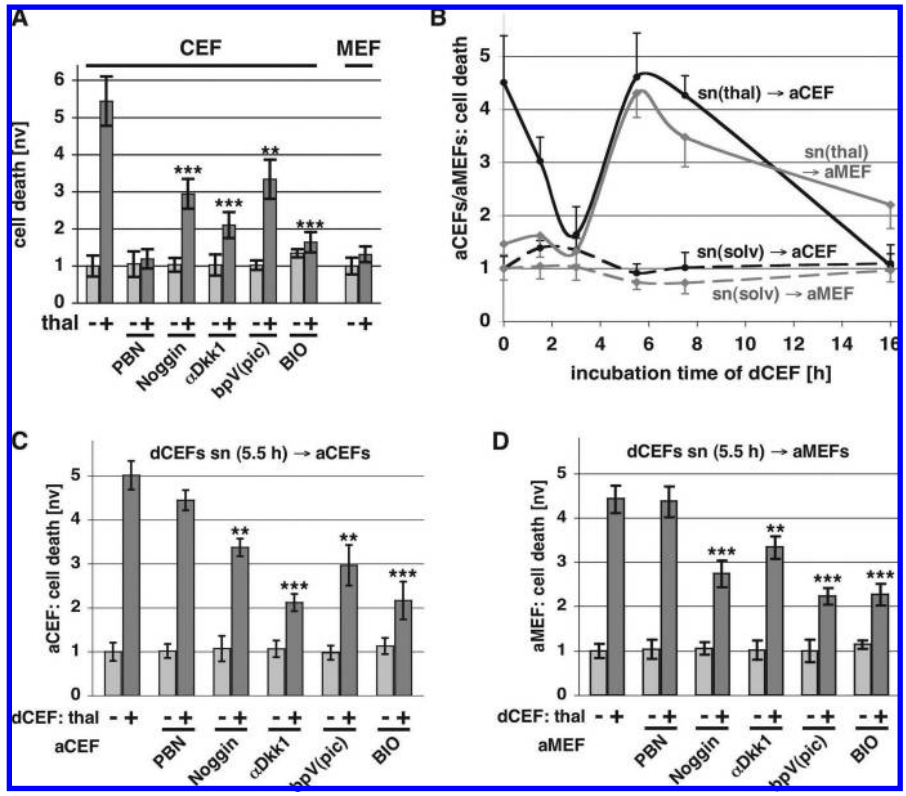

Figure 3. Supernatants of thalidomide-treated CEFs induce cell death in MEFs. (A) CEFs and MEFs were treated with thalidomide and inhibitors as indicated. (B−D) "Donor" CEFs (dCEFs) were treated with thalidomide (thal, +) or a solvent control (−). After the indicated time points the supernatant of thalidomide-treated [sn(thal)] or of solvent-treated [sn(solv)] dCEFs was transferred to "acceptor" CEFs or MEFs (aCEFs, aMEFs) that were preincubated with medium or inhibitors as indicated. After six hours of incubation the ratios of dead to live cells were determined and normalized to the negative controls (CEFs with solvent control or aCEFs/aMEFs with supernatant of solvent-treated dCEFs). A value of 1 corresponds to 1−2.5% cell death. The data represent the mean ± SD from eight individual experiments. *t* test: **, $p < 0.01$; ***, $p < 0.001$ related to thalidomide-treated CEFs (A), sn(thal)-treated aCEFs (C) or sn(thal)-treated aMEFs (D); nv, normalized values.

Discussion

Thalidomide-induced limb truncations are the result of massively up-regulated apoptosis during embryonic limb development. Thalidomide induces apoptosis in embryonic cells via perturbation of both Bmp/Dkk1/Wnt and Bmp/PTEN/Akt signaling[4,5,14] (this study). The sensitivity of a species to the teratogenic properties of thalidomide is reflected by the potential of the drug to induce apoptosis in its embryonic fibroblasts. Accordingly, thalidomide does not induce apoptosis in MEFs but in CEFs and HEFs[4] (this study). Here, we demonstrate that the generation of detectable superoxide in cells exposed to thalidomide is much more pronounced in HEFs than in MEFs and that the latter can be sensitized to thalidomide by GSH depletion. Moreover, we show that the capacity of the GSH-dependent antioxidant defense against thalidomide-induced ROS is higher in embryonic cells of thalidomide-insensitive species than in those of thalidomide-susceptible species. Conversely, experimental increase of GSH levels in HEFs antagonizes thalidomide-induced apoptosis. In good agreement with these data, it has been shown that thalidomide induces ROS in limb bud cells and embryos of thalidomide-sensitive rabbits but not in those of insensitive mice or rats. The overall embryonic redox potential and in particular that of limb buds is much more oxidative in sensitive rabbits than in rats, and rat limb buds possess higher GSH stores than rabbit limb buds.[15] We show here that thalidomide induces apoptosis in sensitized MEFs via the same key molecules (Bmps, Dkk1, PTEN, Gsk3β) that mediate the effects of thalidomide in CEFs, HEFs, isolated chicken limb bud cells and in vivo in chicken limb buds. Accordingly, thalidomide-free supernatants of thalidomide-treated CEFs drive MEFs into apoptosis, and this effect is impaired by blocking the activity of Bmps, Dkk1, PTEN or Gsk3β. This suggests that the supernatants contain pro-apoptotic factors such as Bmps or Dkk1 whose secretion has been induced by thalidomide. We conclude that the molecular switch controlling species specificity of the teratogenic thalidomide effects is upstream of Bmp signaling

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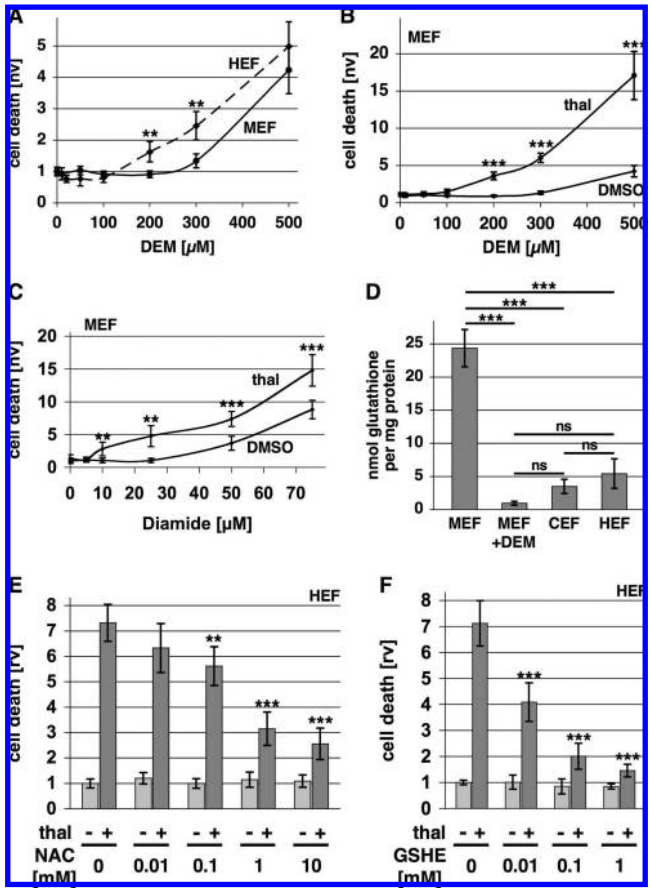

Figure 4. Thalidomide resistance of MEFs depends on the intracellular glutathione level. (A) HEFs or MEFs were incubated with DEM at different concentrations. (B, C) MEFs were incubated with different concentrations of DEM or diamide before addition of a solvent control (DMSO) or thalidomide (thal). (A−C) After six hours of incubation the ratios of dead to live cells were determined and normalized to the negative controls (solvent-treated cells). (D) The total intracellular glutathione content was determined relative to the protein amount of MEFs, of MEFs that were treated with 300 μM DEM for 30 min, of CEFs, and of HEFs. Note that the GSSG content was below 0.1 nmol GSSG/mg protein in all cases (not shown). (E, F) HEFs were preincubated with different concentrations of *N*-acetyl-cysteine (NAC) or GSH ethyl ester (GSHE) and then treated for six hours with solvent or thalidomide. Cell death was determined as in A−C. The data represent the mean ± SD (±SEM in D) from eight (A, B, D) or six (C, E, F) individual experiments. A value of 1 corresponds to 1−2.5% cell death. *t* test: **, $p < 0.01$; ***, $p < 0.001$ related to MEFs (A) or DMSO-treated MEFs (B, C) at the same respective DEM concentration or to thalidomide-treated HEFs that were not preincubated with NAC or GSHE (E, F); nv, normalized values; ns, not significant.

and essentially a consequence of differences between cell types at the level of superoxide production. This in turn may be the result of differences in antioxidant defense that is

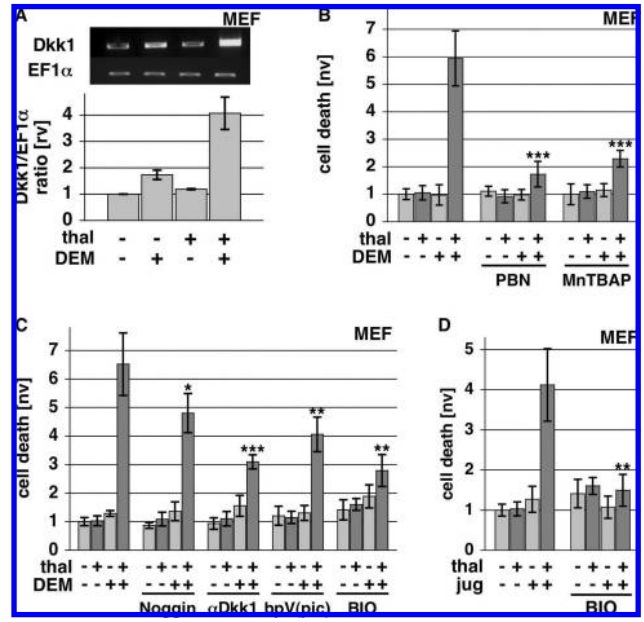

Figure 5. Blocking of Bmps, Dkk1, PTEN or Gsk3β prevents thalidomide-induced apoptosis in sensitized MEFs. MEFs were preincubated with DEM (300 μM), juglone (100 nM) or a solvent control (DMSO) and inhibitors as indicated before addition of thalidomide (thal, 38.7 μM) or a solvent control (DMSO). After six hours of incubation Dkk1 or EF1α (control) mRNA levels were determined by semiquantitative RT-PCR (A) or the ratios of dead to live cells were determined and normalized to the negative controls (solvent-treated cells) (B–D). (A) One representative set of RT-PCRs and the densitometric evaluation of two independent experiments is shown. (B–D) The data represent the mean ± SD from six (B, D) or eight (C) individual experiments. A value of 1 corresponds to 1−2.5% cell death. *t* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ related to thalidomide/DEM- (B, C) or to thalidomide/juglone-treated (D) cells; nv, normalized values.

required for neutralization of thalidomide-induced oxidative stress during embryogenesis.

Our data suggest that thalidomide-induced apoptosis is mainly mediated by superoxide as well as by an as yet unspecified extent of hydrogen peroxide generated under the influence of thalidomide. This is in line with the fact that the generation of hydrogen peroxide in the presence of thalidomide has previously been demonstrated.[16] But how does superoxide trigger cell death in embryonic cells? Thalidomide suppresses Wnt and Akt survival pathways by the up-regulation of Bmp expression.[4,5] Nuclear factor κB (NF-κB) is a redox-sensitive transcription factor and key regulator of embryonic limb outgrowth. Thalidomide-induced ROS were hypothesized to impair DNA binding of NF-κB

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through the oxidation of a cysteine within the DNA binding domain.[15] Interestingly, NF-κB is a negative regulator of Bmp signaling,[17] and treatment of mice with a superoxide scavenger results in decreased Bmp4 expression.[18] Thus, superoxide generated by thalidomide might impair the repressor activity of NF-κB on Bmp expression in embryonic cells.

In summary, our study shows a molecular basis for thalidomide insensitivity of mouse embryonic cells implying that depletion of the antioxidant defense may be a promising strategy to develop thalidomide-sensitive mice. These mice would allow for research on thalidomide-induced molecular pathology during embryogenesis in vivo with high relevance for humans. Our findings might also be useful for the development of thalidomide derivatives that lack teratogenic activity but still keep their beneficial properties with regard to cancer and inflammation-based diseases.

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