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Thalidomide is an inhibitor of angiogenesis

(fibroblast growth factor/rabbit cornea)

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ABSTRACT **Thalidomide is a potent teratogen causing dysmelia (stunted limb growth) in humans. We have demonstrated that orally administered thalidomide is an inhibitor of angiogenesis induced by basic fibroblast growth factor in a rabbit cornea micropocket assay. Experiments including the analysis of thalidomide analogs revealed that the antiangiogenic activity correlated with the teratogenicity but not with the sedative or the mild immunosuppressive properties of thalidomide. Electron microscopic examination of the corneal neovascularization of thalidomide-treated rabbits revealed specific ultrastructural changes similar to those seen in the deformed limb bud vasculature of thalidomide-treated embryos. These experiments shed light on the mechanism of thalidomide's teratogenicity and hold promise for the potential use of thalidomide as an orally administered drug for the treatment of many diverse diseases dependent on angiogenesis.**

Thalidomide is a potent teratogen. It was developed by Chemie Grunenthal in the 1950s as a sedative that appeared so nontoxic in rodent models that a LD_{50} could not be established. In 1961, McBride (1) and Lenz (2) described the association between limb defects in babies and maternal thalidomide usage. Although humans are exquisitely sensitive to the teratogenic effects of thalidomide, experiments in rodents failed to reveal similar effects (3, 4). Teratogenic effects could be experimentally reproduced by the administration of thalidomide to pregnant rabbits at an oral dose of 100–300 mg per kg per day (5, 6). Over the past 30 years, the mechanism of thalidomide's teratogenicity has been extensively studied but has remained unsolved (7).

We now postulate that the limb defects seen with thalidomide were secondary to an inhibition of blood vessel growth in the developing fetal limb bud. The limb bud is unique in requiring a complex interaction of both angiogenesis and vasculogenesis during development (8). Vasculogenesis is the formation of a capillary bed from endothelial cells that have differentiated from mesenchymal precursors. Angiogenesis is the formation of new blood vessels from sprouts of preexisting vessels. Therefore, the limb bud would be a particularly vulnerable target to a teratogen that inhibited endothelial cell function. We chose to examine the effect of thalidomide on growing vasculature in the chicken chorioallantoic membrane and in the rabbit cornea.

MATERIALS AND METHODS

Chicken chorioallantoic membrane (CAM) assays were performed as described (9, 10) and the effects on the developing vasculature were recorded at 48 h after implantation of the 0.5% carboxymethylcellulose pellet containing various drugs. Corneal neovascularization was induced by an implanted pellet of poly(hydroxyethyl methacrylate) (Hydron; Interferon Sciences, New Brunswick, NJ) containing 650 ng of the potent angiogenic protein basic fibroblast growth factor (bFGF) bound to sucralfate (sucrose aluminum sulfate; Bukh Meditec, Copenhagen) (11). The addition of sucralfate to the pellet protects the bFGF from degradation (12) and provides for its slow release, thus producing consistent aggressive angiogenesis that is more pronounced than that induced by bFGF/Hydron alone. Release of bFGF from pellets containing sucralfate/Hydron could be detected *in vitro* for up to 4 days after the pellets were formed compared to just 1 day for pellets with Hydron alone (11). Pellets were made by mixing 110 μl of saline containing 12 μg of recombinant bFGF (Takeda, Osaka) with 40 mg of sucralfate; this suspension was added to 80 μl of 12% (wt/vol) Hydron in ethanol. Aliquots (10 μl) of this mixture were then pipetted onto Teflon pegs and allowed to dry producing approximately 17 pellets. A pellet was implanted into corneal micropockets of each eye of an anesthetized female New Zealand White rabbit, 2 mm from the limbus, followed by a single topical application of erythromycin ointment on the surface of the cornea. Histologic examination on consecutive days demonstrated progressive blood vessel growth into the cornea toward the pellet with only rare inflammatory cells seen. This angiogenic response was not altered by severe immune suppression with total body irradiation, and pellets with sucralfate alone did not induce angiogenesis (data not shown). Unlike other models of corneal angiogenesis that utilize inflammation to stimulate neovascularization, the new vessels are primarily induced by the bFGF. The animals were fed daily from 2 days after implantation by gastric lavage with either drug suspended in 0.5% carboxymethylcellulose or vehicle alone. Thalidomide was purchased from Andrulus Pharmaceutical (Beltsville, MD) and EM-12 and Supidimide were kindly provided by Grunenthal (Stolberg, F.R.G.). Immunosuppressed animals received total body radiation of 6 Gy for 6 min immediately prior to implantation of the pellets. This dose of radiation resulted in a marked leukocytopenia with >80% reduction in the leukocyte count by day 2 and >90% reduction by day 3, results that are consistent with previous reports (13, 14).

The animals were examined with a slit lamp every other day in a masked manner by the same corneal specialist (M.S.L.). The area of corneal neovascularization was determined by measuring with a reticule the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. A formula was used to determine the area of a circular band segment: $C/12 \times 3.1416 [r^2 - (r - L)^2]$, where $r = 6$ mm, the measured radius of the rabbit cornea. We have utilized various mathematical models to determine the amount of vascularized cornea and have found that this formula provides the most accurate approximation of the area of the band of neovascularization that grows toward the pellet. Only the uniform contiguous band of neovasculariza-

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Abbreviations: bFGF, basic fibroblast growth factor; CAM, chicken chorioallantoic membrane; PGA, phthaloylglutamic anhydride; PG acid, phthaloylglutamic acid; TNF-α, tumor necrosis factor α.

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tion adjacent to the pellet was measured. The noncontiguous neovascularization, which can be seen superiorly, was not quantified due to its irregular shape. These vessels that often grow concurrently toward the pellet from the superior limbus arise from vessels of the superior rectus supplying the limbus, are directly induced by the bFGF/sucralfate pellet, and are histologically identical to the inferior limbal vessels. However, it should be noted that this superior neovascularization was commonly seen in control animals and was never seen in thalidomide-treated animals. Thus, the total inhibition of neovascularization is conservatively underestimated.

RESULTS

Our initial investigations were performed on the CAM. Neither thalidomide nor EM-12, a related teratogenic analog (15), exhibited any inhibitory activity on blood vessel growth. This result was expected as it has been proposed that thalidomide must be metabolized by the liver to form an epoxide that may be the active teratogenic metabolite (16). Other thalidomide analogs that have been shown to be teratogenic in rodents (17), including phthaloylglutamic anhydride (PGA) and phthaloylglutamic acid (PG acid), were also analyzed (Fig. 1). Interestingly, weak antiangiogenic activity of the developing vasculature was seen with both PG acid and PGA when 100 μg of either compound was placed on the CAM in a pellet of 0.5% carboxymethylcellulose. Despite frequent mild scarring, avascular zones were produced in 15% of the CAMs with PGA compared to control 0.5% carboxymethylcellulose pellets in which no avascular zones were seen (data not shown).

Based on these initial findings, we decided to test thalidomide's effect on angiogenesis induced by bFGF in the rabbit corneal micropocket model. Treatment with a teratogenic dose (200 mg/kg) of thalidomide resulted in an inhibition of the area of vascularized cornea that ranged from 30 to 51% in three experiments with a median inhibition of 36% (Figs. 2*A* and 3) (n = 30 eyes; P = 0.0001, two-way ANOVA with ranked data). The inhibition of angiogenesis by thalidomide was seen after only two doses (Fig. 2*B*). The rabbits did not demonstrate obvious sedation and there were no signs of toxicity or weight loss. The teratogenic analog EM-12, which shares the other properties of thalidomide, was also inhibitory, with a median inhibition of 42% (n = 10 eyes; P = 0.002, one-way ANOVA with ranked data). Supidimide, a nonteratogenic analog that retains the sedative properties of thalidomide, exhibited no activity (area 107% of control; n = 10 eyes; not statistically different from control). Other analogs, PGA and PG acid, displayed weaker inhibitory effects

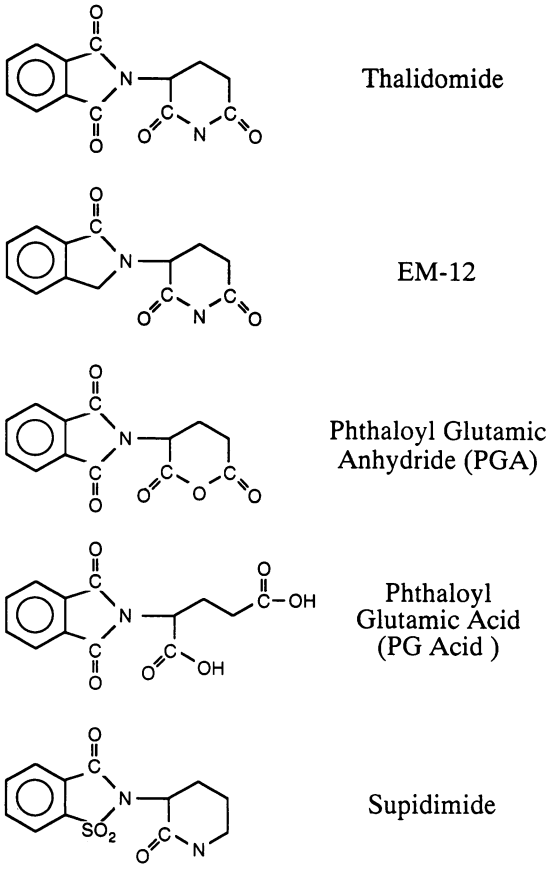

FIG. 1. Structure of thalidomide and related analogs.

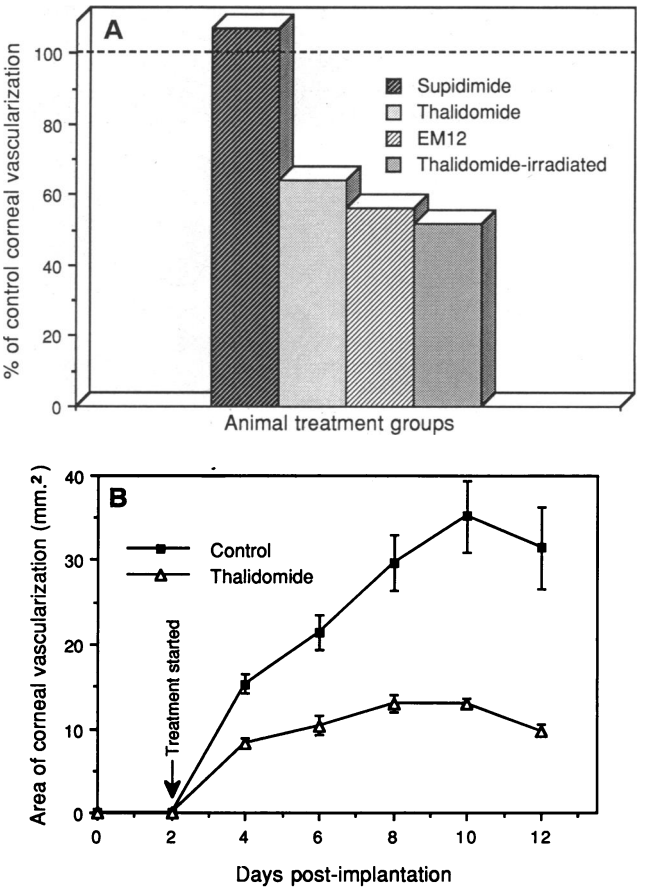

FIG. 2. (*A*) Inhibition of bFGF-induced corneal neovascularization by thalidomide and related analogs expressed as percent of median control on day 8. Pellets containing bFGF and sucralfate were implanted into micropockets of both corneas of rabbits (18). Vessel ingrowth into the clear cornea from the limbus was first noted on day 2 and treatments (200 mg/kg orally) were begun on this day. The area of corneal neovascularization was measured from day 4 through day 12. Day 8 measurements were used for comparison between groups. No regression of vessels and near maximal neovascularization was seen at this time point. Statistical analysis was performed with ANOVA with ranked data to account for interexperimental variation and to guard against a nonnormal distribution of data (i.e., outliers) by utilizing a nonparametric method. (*b*) Time course of inhibition of neovascularization with thalidomide. Mean areas of corneal neovascularization with standard error bars are presented from one experiment that is representative of the three experiments performed with thalidomide on nonirradiated animals. Data presented from the first time point after administration of the drug through the completion of the study are statistically different (n = 10 eyes; $P < 0.005$ for all time points, one-way ANOVA with ranked data).

than thalidomide (data not shown). The density of vessel ingrowth in thalidomide-treated animals was also markedly reduced. Due to the lack of an objective grading scale, these results are not presented.

Thalidomide has immunosuppressive properties that might have indirectly affected angiogenesis. Recently, thalidomide has been used for its immunosuppressive properties in the treatment of leproma reactions (19) and chronic graft versus host disease (18, 20–23). However, in humans its immunosuppressive properties are weak and delayed with little effect in acute graft versus host disease (24). Because the effect of thalidomide on the immune system is similar but weaker than that of cyclosporin A (25), we tested cyclosporin A at the highest tolerated dose of 25 mg/kg. No statistically significant effect was observed compared to control. To investigate further the immune interactions, we pretreated the rabbits with the maximally tolerated immunosuppressive dose of total body irradiation. Immunosuppressed animals responded equally well to thalidomide, with a median inhibition of neovascularization of 52% ($n = 12$; $P = 0.0001$, one-way ANOVA with ranked data) as compared to irradiated placebo-treated controls (Fig. 2*A*).

Electron microscopic examination of corneas from thalidomide-treated and control animals revealed ultrastructural differences. Vessels in the thalidomide-treated group dem-

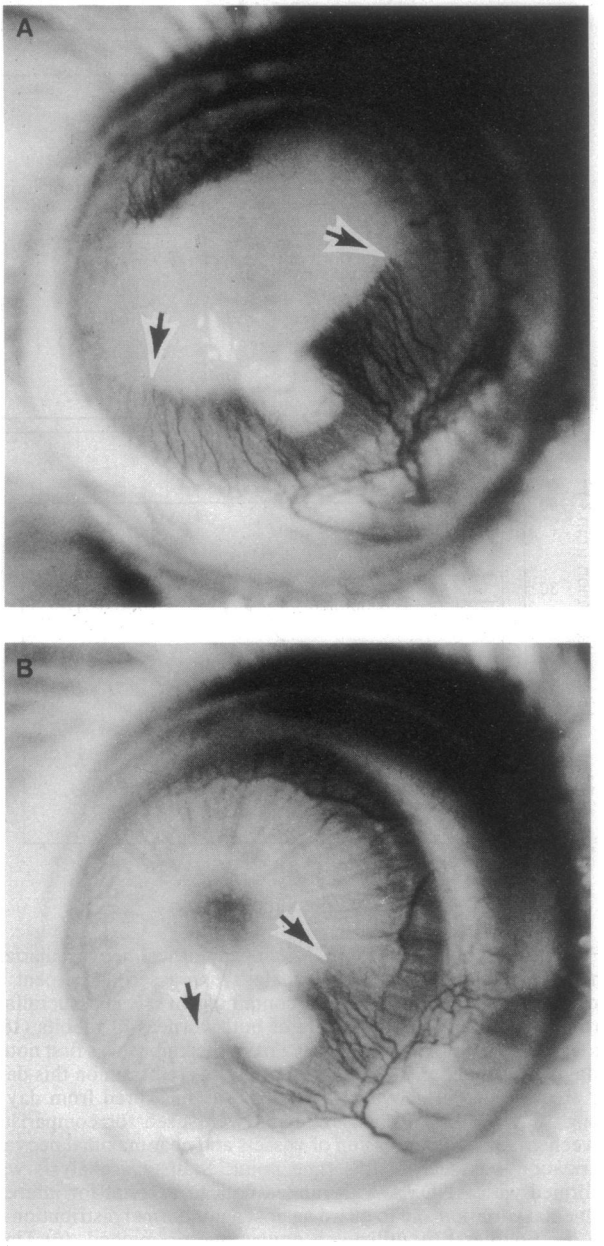

FIG. 3. Representative corneas at 8 days after implantation of bFGF pellets from control (*A*) and thalidomide-treated (*B*) rabbits. There is prominent corneal neovascularization (arrows) in the control with associated corneal clouding, which was demonstrated histologically to be stromal edema without inflammation. The thalidomide-treated animal has markedly less neovascularization with minimal corneal edema.

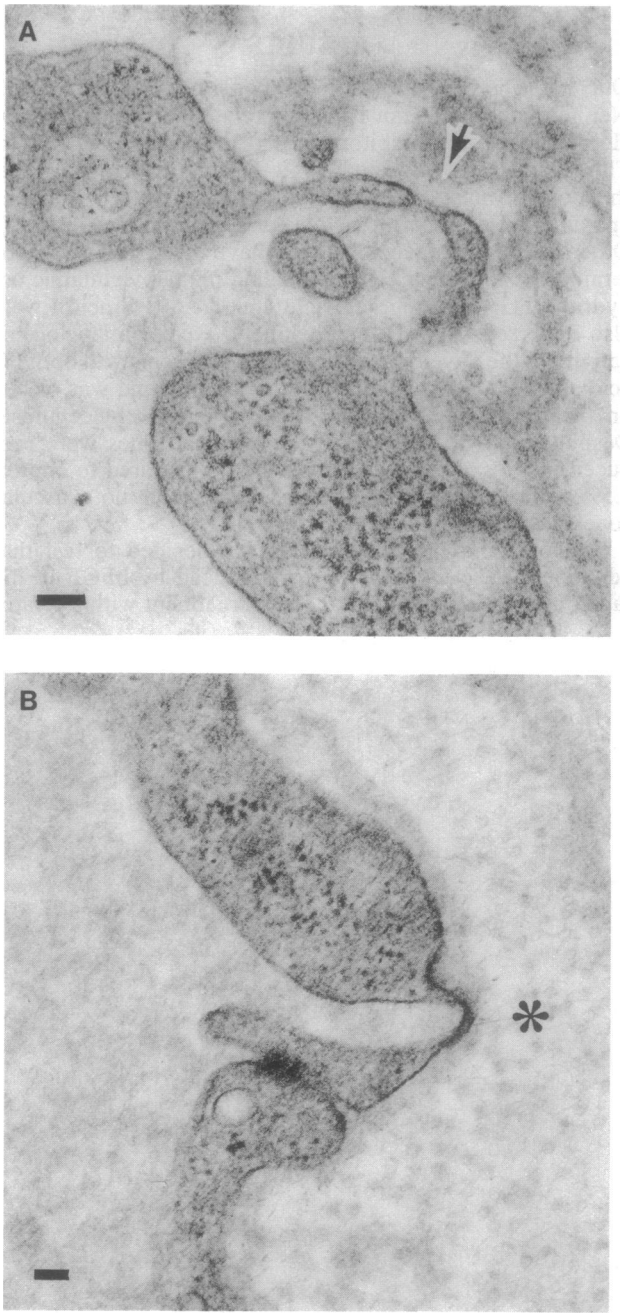

FIG. 4. Electron micrographs of corneal neovascularization observed in a thalidomide-treated rabbit 10 days after implantation of a pellet containing bFGF. (*A*) High-magnification (×40,000) view of typical fenestrations (arrow) in an endothelial cell from corneal neovascularization in thalidomide-treated rabbit. (*B*) High-magnification (×60,000) view of an area of cell thinning (asterisk) adjacent to a cell junction in thalidomide-treated corneal neovascularization. These changes were not seen in control day 10 corneal neovascularization. (Bars = 0.1 μm.)

onstrated fenestrations not seen in control animals (Fig. 4*A*). Fenestrations have been previously reported to be specific to regressing corneal blood vessels after removal of the angiogenic stimulus (26). However, in that model, endothelial cell regression was associated with platelet plugging and cellular hypoxic changes such as swollen mitochondria, which were not seen in the thalidomide-treated animals. Interestingly, histologic changes previously described in the vasculature of the limb buds from chicken embryos treated with thalidomide (27) were also seen in the corneal neovascularization of our thalidomide-treated rabbits including vesicular projections into the lumen and extreme thinning of cell processes (Fig. 4*B*). In general, the corneal neovascularization from thalidomide-treated rabbits appeared more immature than that observed in control animals with poorly formed cell junctions, incomplete basement membrane, and fewer associated pericytes. These findings support the hypothesis that thalidomide has a direct effect on the growing vasculature.

DISCUSSION

Orally administered thalidomide is an inhibitor of angiogenesis induced by bFGF in the rabbit cornea micropocket assay. The mechanism by which thalidomide inhibits angiogenesis is unknown. Thalidomide has shown no effect on bFGF-induced proliferation of endothelial cells in culture (data not shown). Current studies are focused on the identification of the most active thalidomide metabolite. The formation of an active metabolite by the liver *in vivo* provides an explanation of the observation that the effect of thalidomide on growing vessels is seen only when given systemically.

Thalidomide has been shown to suppress tumor necrosis factor α (TNF-α) production from macrophages (28). However, macrophages were rarely seen in histologic examinations of our model of corneal neovascularization. Furthermore, studies examining the role of TNF-α in corneal angiogenesis have failed to detect TNF-α production in a model of inflammatory corneal angiogenesis in which macrophages were prominent (29). TNF-α is only weakly angiogenic *in vivo*. It acts by inducing secondary inflammation in contrast to bFGF, which stimulates angiogenesis without inflammation (30). Thus, the ability of thalidomide to inhibit angiogenesis induced by pharmacologic doses of bFGF supports the hypothesis that thalidomide directly inhibits an essential component of angiogenesis and does not operate through effects on TNF-α production.

In conclusion, thalidomide is a potent angiogenesis inhibitor *in vivo*. In this model of corneal angiogenesis, we have tested many putative angiogenesis inhibitors (including antimitotic agents, *cis*-retinoic acid, tamoxifen, and others). Thalidomide was the only agent capable of inhibiting angiogenesis after oral administration. Evaluation of thalidomide analogs demonstrated a correlation between teratogenicity and antiangiogenic potential. The weak and delayed immunosuppressive action of thalidomide when used clinically, its inhibition of angiogenesis in radiation-immunosuppressed animals, and the lack of effect of the functionally related immunosuppressive agent cyclosporin A argue for a direct effect of thalidomide on angiogenesis. Further support for this hypothesis is derived from the ultrastructural changes seen in thalidomide-treated animals. There are clear implications for the use of this drug in the treatment of pathologic angiogenesis that occurs in diabetic retinopathy, macular degeneration, and solid tumors. Because antiangiogenic therapy is likely to be long-term, there is a need for an orally efficacious inhibitor.

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