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Thalidomide inhibits corneal angiogenesis induced by vascular endothelial growth factor

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Abstract ● Background: Ocular diseases caused by neovascularization are among the leading causes of blindness. No specific pharmacological treatment is available. Among potential drugs, thalidomide deserves special interest since a wide body of clinical experience exists. However, its antiangiogenic effect is controversial. We therefore investigated the effect of thalidomide on corneal angiogenesis induced by vascular endothelial growth factor (VEGF), which has a special role among angiogenic growth factors. ● Methods: Corneal neovascularization was induced in NZW rabbits by an intrastromal pellet loaded with 500 or 750 ng VEGF. Animals received two daily feedings of 200 mg/kg thalidomide. ● Results: Significant inhibition of corneal angiogenesis ($P<0.0001$) was caused by the teratogenic dose of thalidomide after the 5th day of treatment and persisted for more than 16 days. No obvious side effects were recorded. ● Conclusions: Thalidomide has a significant antiangiogenic effect against VEGF-induced neovasclar growth. Together with earlier findings this observation indicates that the drug inhibits two angiogenic pathways which are mediated through integrin adhesion molecules.

Introduction

Diseases which are characterized by neovascular growth are amongst the most challenging therapeutic problems in clinical ophthalmology. While certain forms of ocular neovascularization are susceptible to laser treatment or surgical interventions there is still no specific pharmacological antiangiogenic therapy available for clinical use. During the last years numerous investigations primarily in the context of tumor angiogenesis have revealed a wide body of information concerning the mechanisms which underlay the formation of new blood vessels [20]. Also, various substances have been identified which inhibit several aspects of neoangiogenesis both in vitro and in animal experiments [15]. One of these substances, interferon-alpha 2a, has recently been used in patients in the context of choroidal neovascularization associated with age-related macular degeneration [6], following its successful use for the treatment of vascular tumors in infants [27]. This has led to the first clinical trial evaluating a pharmacological antiangiogenic treatment in ophthalmology.

Although animal experiments suggest that several substances could be used to treat ocular neovascularization in humans, unknown and potentially severe side effects preclude clinical application in most cases. However, there are several drugs with antiangiogenic potential which have been used for other purposes in humans and whose side effects are therefore well known. One of these drugs is thalidomide, an immunomodulatory drug which acts as a potent teratogen in humans as well as non-human primates. However, if applied to the non-pregnant patient it has very few side effects apart from its sedative and neurotoxic properties, and it is therefore currently used for various special indications in humans. The report by D'Amato and coworkers that thalidomide can halt the formation of experimentally induced corneal neovascularization therefore introduced the hope that a relatively safe

drug can be utilized for the treatment of ocular neovascular diseases [4]. However, in the meantime several reports have cast a shadow of doubt upon the value of thalidomide's antiangiogenic properties. Several animal experiments [12, 25] as well as a case report [9] failed to reproduce the inhibitory effect on the growth of newly developing blood vessels.

In addition, more recent reports on the mechanism of corneal angiogenesis have revealed at least two distinct pathways characterized by certain integrins which are induced by different polypeptide growth factors. One pathway is dependent on the integrin $\alpha_V\beta_3$ and is induced by fibroblast growth factor (bFGF), whereas the second pathway is dependent on $\alpha_V\beta_5$ and can be induced by vascular endothelial growth factor (VEGF) [5]. In the original report which initially described the antiangiogenic properties of thalidomide, the authors utilized bFGF to initiate the growth of blood vessels into the rabbit cornea [4] and therefore tested the effect of the drug on the pathway through $\alpha_V\beta_3$ integrins. However, the growth of human ocular neovascularization seems to be largely dependent on the formation of VEGF, which induces the pathway mediated through $\alpha_V\beta_5$. Therefore, the present study investigated the effect of thalidomide on corneal angiogenesis induced by VEGF.

Methods

Induction of corneal vessels by VEGF

Experiments were conducted in New Zealand White (NZW) rabbits according to the resolution of the use of animals in research as published by the Association for Research and Vision in Ophthalmology (ARVO) and were performed under observation of German federal laws. All surgical procedures, as well as the quantification of the newly developed blood vessels, were performed under intramuscular general anesthesia [xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (35 mg/kg)].

A corneal micropocket assay was used for the induction of corneal blood vessels in response to VEGF administered intrastromally. In order to achieve a continuous stimulus for several days the growth factor has to be incorporated into a carrier substance. To by-pass potential problems related to the degradation of protein growth factors in carrier substances which contain ethanol during preparation, we chose methylcellulose as carrier substance for VEGF. In slight modification of the method described by Crum et al. [3], 50 µl of 2% methylcellulose (4000 centipoise; Sigma Chemie, Deisenhofen, Germany) was carefully placed on a plastic rod with a diameter of 4 mm and allowed to dry under a tissue culture hood after being loaded with either 500 or 750 ng human recombinant VEGR (R&D, Minneapolis, Minn. USA). Dry pellets were folded and used within several hours. For intrastromal implantation a horizontal cut was made in the center of the cornea and a stromal tunnel created by help of a crescent rounded blade (G-34080, Geuder, Heidelberg, Germany) towards the 12'o clock position. Following implantation the entrance of the tunnel was closed with a single 10-0 nylon suture to prevent uncontrolled liberation of the growth factor. The distance between the peripheral margin of the pellet, which is equivalent to the peripheral limit of the tunnel, and the limbus cornea was 2.5 mm. Earlier experiments showed that this experimental setup does not lead to the induction of corneal blood vessels when pellets without growth factors are used [11]. Animals were observed daily,

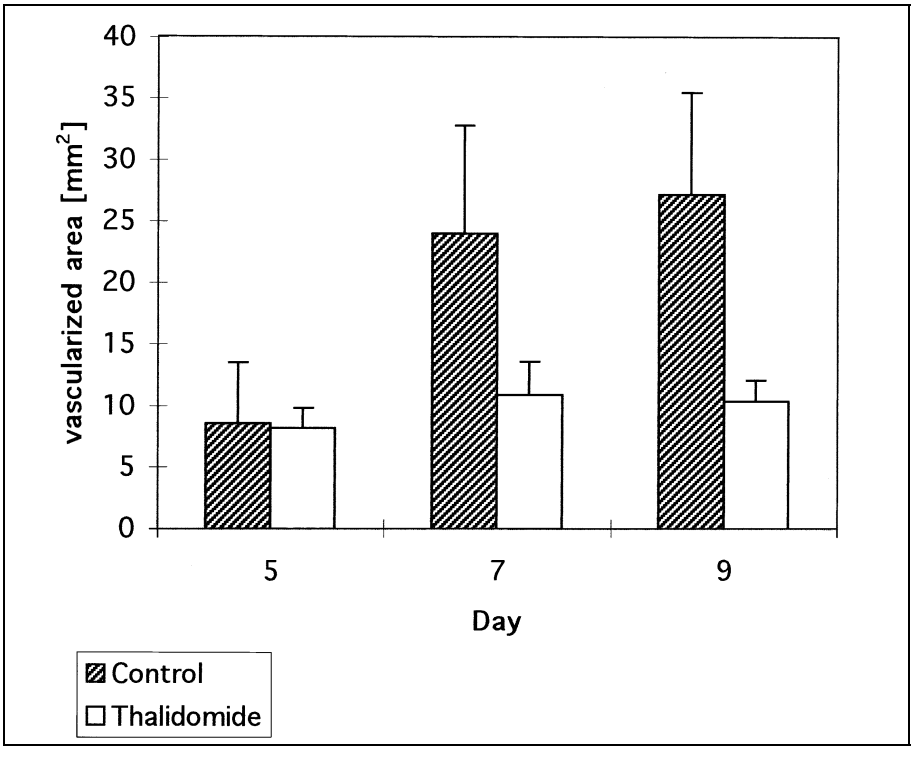

Fig. 1 Mean vascularized area in response to intracorneal pellet loaded with 500 ng rh-VEGF. Seven rabbits received 200 mg/kg thalidomide twice daily, while control animals only received solvent

and vascular growth was quantified under the operating microscope on day 3, 5, 7, 9, 12 and 16 after surgery. The vascularized area was calculated from measurements performed both in vivo (by use of a caliper under the microscope) and on standardized photographs. Differences between treated animals and controls were tested using unpaired Student's *t*-test. In addition, the density of the vessels at the limbus was quantified. The very high density of the vessels at the central limitation of the vascularized area (vessel front) precluded a reliable numerical quantification.

Treatment with thalidomide

Thalidomide (Grünenthal, Aachen, Germany) was solved in 0.5% carboxymethylcellulose (Fluka, Buchs, Switherland) and administered by gastric lavage. This preparation was made freshly prior to each individual feeding. In order to mimic treatment modalities in humans, where the drug is given twice daily, animals received thalidomide every 12 h starting from the evening of the day of surgery. A total of 14 animals (four separate experiments) with pellets containing VEGF (either 500 ng or 750 ng) received two doses of 200 mg/kg per day while an equal number of rabbits (control group) received only 0.5% carboxymethylcellulose without the drug.

Results

On the 2nd day following the implantation of pellets containing VEGF, moderate dilatation of vessels of the limbus as well as the iris was observed. On day 3 initial sprouting of new blood vessels occurred. Continuous vascular growth was observed thereafter.

Treatment of rabbits with thalidomide did not result in significant side effects such as sedation, signs of neurotoxicity or weight loss. A marked retardation of the growth of blood vessels was observed when animals were treated with a teratogenic dose of 200 mg/kg twice daily. However, this retardation was not obvious in the early phase of neovascular growth: On days 3 to 5 no statistically significant difference in vascular growth could be detected between treated and untreated animals when pellets with 500 ng VEGF were used as stimulus. As shown in Fig. 1 the mean vascularized area was not significantly different between treated animals and the controls on the 5th day after surgery (8.1±1.7 vs 8.6±4.9 mm^2 in the control group). Therefore, however, significant retardation of the vascular growth was observed among animals receiving thalidomide. As also shown in Fig. 1, neovascular growth was inhibited by 55% on day 7 and by 62% on day 9. The difference in the vascularized area between animals treated with thalidomide and the controls was statistically significant at both time points ($P<0.0001$, $n=7$, unpaired *t*-test). Interestingly, there was almost no increase in the vascularized area between day 7 and day 9 among animals treated with thalidomide, while the area increased by about 14% in the controls. The density of the vessels at the limbus was not significantly different between treated animals and controls (Fig. 2). The quantification of vessels at the limbus did also not show a significant difference (data not shown). The density of vascular sprouts

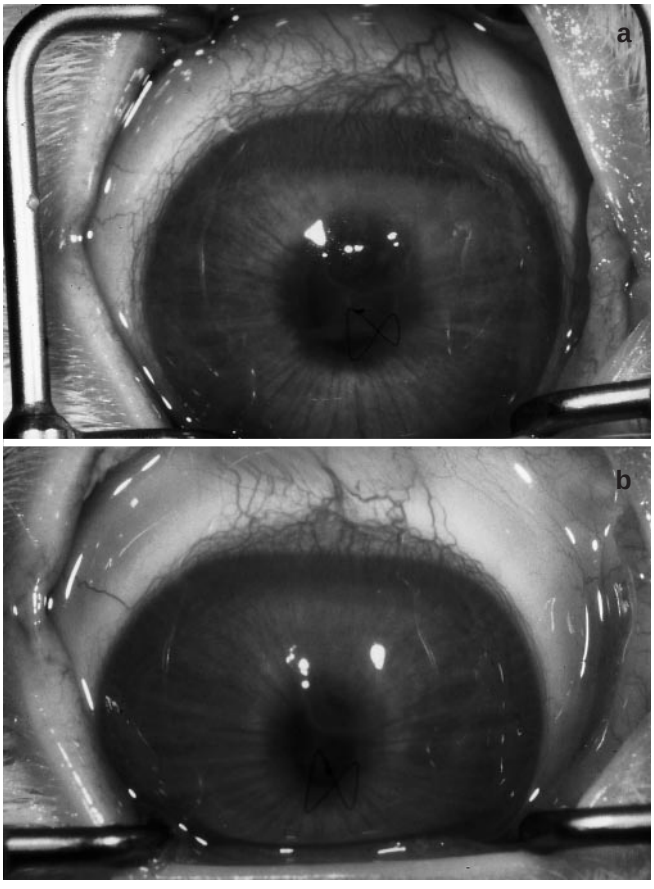

Fig. 2 Representative corneas 7 days after implantation of VEGF pellets from **a** a control rabbits and **b** a thalidomide-treated rabbit. The treated rabbit was significantly less neovascular growth

at the leading edge of the vascularized area towards the pellet was equally high (Fig. 2). Furthermore we did not observe any difference in the ratio of deep to superficial vessels between rabbits treated with thalidomide and the controls.

To investigate whether thalidomide could also inhibit the growth of vessels when the size of the stimulus was increased, we performed a second set of experiments with pellets loaded with 750 ng of VEGF. Similar to the first set of experiments with 500 ng VEGF the difference in neovascular growth was not significant prior to day 5 (data not shown). However, on the following days a significant inhibition could be observed which was apparent on biomicroscopical observation of the animals (Fig. 2). Quantification of the neovascular growth (Fig. 3) revealed a statistically significant inhibition of neovascular growth on days 7 and 9 ($P<0{,}0001$, $n=7$, unpaired *t*-test). In addition, observation of the eyes of days 12 and 16 disclosed inhibition of a magnitude (62% and 61%) comparable to that observed on day 9.

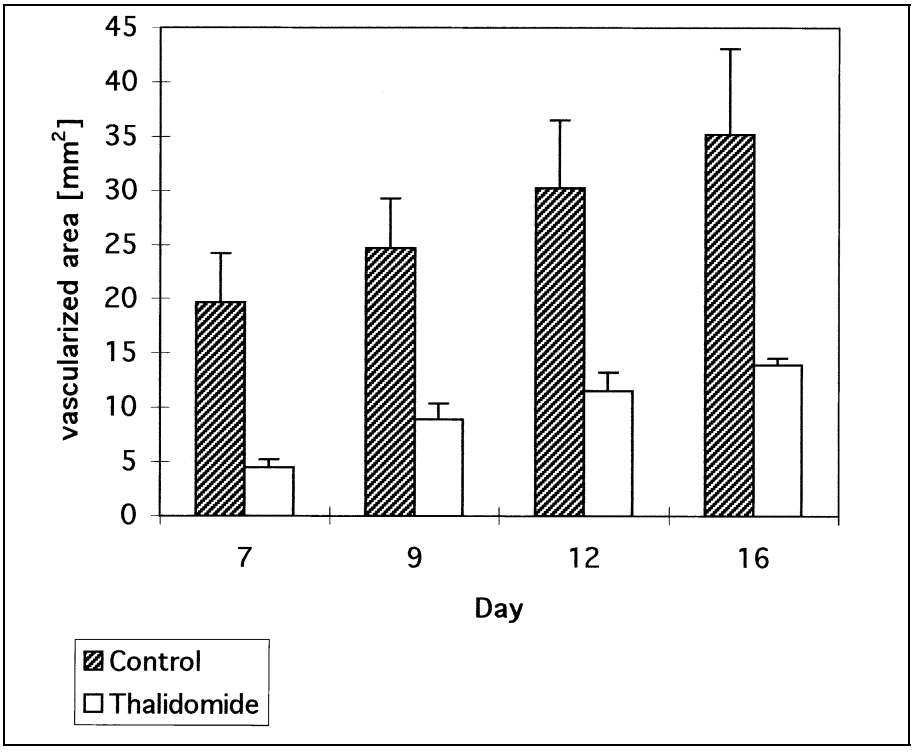

Fig. 3 Mean vascularized area in response to an intracorneal pellet loaded with 750 ng rh-VEGF. Seven rabbits received 200 mg/kg thalidomide twice daily, while control animals only received solvent

Discussion

Our results confirm that orally administered thalidomide is an inhibitor of angiogenesis. Furthermore, the results are in concordance with the observations made by D'Amato and coworkers, who reported inhibition of the vascularized area of up to 51% when bFGF was used as angiogenic stimulus [4]. Similarly the vascularized area in treated animals did not increase between day 8 and 12, a finding which was also confirmed by our data. It can be concluded that thalidomide inhibits two pathways of neo-angiogenesis, one mediated through integrin $\alpha_V\beta_3$ and the other through integrin $\alpha_V\beta_5$.

However, our results are in discordance with three other reports indicating that thalidomide is not able to cause a significant effect on the growth of blood vessels. These discrepancies could be explained by differences in the experimental design in the case of one study [12] which showed a marked inflammatory response with marked cellular infiltration in the vicinity of the pellet. Such a response, which was not observed in our animals, could have changed the microenvironment in the cornea, especially since inflammatory cells can mediate potent angiogenic stimuli [24]. Also, special care must be taken during the preparation of thalidomide, which might lose some of its bioactivity on prolonged storage. The discrepancy from the second study might primarily depend on species differences, since rats, which were used by Tsujikawa and coworkers [25], are far less susceptible to the teratogenic effects of thalidomide. Finally, the recent observation of persisting neoangiogenic growth in humans under thalidomide treatment can only be tested for its validity in a larger population of human patients [9]. The results of such a study, which is currently underway, may help to determine the use of thalidomide's antiangiogenic effect for the treatment of ocular neovascularization.

The mechanisms which underlie the obvious antiangiogenic effect in the rabbit cornea are currently unknown. In the recent past thalidomide has been used as an immuno-modulatory drug and has increased the survival rates of bone marrow and organ transplants both in animal experiments and in humans [19, 26]. Furthermore, several immunological disorders, such as rheumatoid arthritis [8], Behcet's syndrome [13] and lupus erythematosus [2] have been successfully treated in humans. In ophthalmology the effect of thalidomide was investigated in the context of experimental uveitis in rats [7]. One of the underlying mechanisms for this immunomodulatory effect is the reduction of the synthesis of tumor necrosis factor-alpha (TNF-α) both at the level of inflammatory cells, such as monocytes [21], and at the serum levels of patients, e.g. in the context of leprosy [22]. Whether or not the suppresion of TNF-α is also relevant to the inhibition of corneal angiogenesis is debatable and needs further investigation. Circumstantial evidence suggests that TNF-α plays a rather moderate role in experimental corneal angiogenesis; this inflammatory cyto-

kine was not prominent even in the context of inflammation [24], and thalidomide was also able to inhibit corneal angiogenesis in leukopenic animals which had been irradiated [4]. Besides TNF-α, thalidomide can modulate other cytokines associated with neovascular growth: it has been shown to enhance the production of interleukin-4 and -5, and it significantly inhibits interferon-gamma in peripheral blood mononuclear cell cultures [14]. Taken together, these results suggest that one aspect of thalidomide's antiangiogenic mechanism might be related to a modulation of cytokines. A second aspect concerning thalidomide's antiangiogenic mechanism might be related to the expression of cell adhesion molecules. The importance of cell surface receptors and the contact to extracellular structures such as the basement membrane is stressed by the above-mentioned significance of integrins for neoangiogenesis. Investigations performed by Neubert and coworkers have shown that thalidomide and some of its derivatives can change the pattern of integrins and cell surface receptors on mammalian blood cells [16–18]. Especially adhesion molecules such as LFA 1 or 2, as well as ICAM-1, are modulated by thalidomide. It has been shown that adhesion molecules, e.g. VCAM-1 or E-selectin, play a significant role in early angiogenesis since they modulate the recruitment of inflammatory cells [10]. By use of intravital microscopy we have recently shown that a significant increase in the number of leukocytes adhering to the limbal vascular endothelium occurs in the early phase of corneal angiogenesis induced by VEGF [1]. Schneider and coworkers have used a similar method to show that thalidomide slows the extravasation of leukocytes [23]. These findings suggest that alteration of extracellular adhesion molecules also plays a role in experimental corneal angiogenesis. Taken together, these observations indicate that modulation of surface receptors might be a mechanism by which the effect of thalidomide on experimental corneal angiogenesis is mediated.

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