

LABORATORY SCIENCE

DMSO mimics inhibitory effect of thalidomide on choriocapillary endothelial cell proliferation in culture

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Aim: To evaluate the effect of thalidomide on bovine choriocapillary endothelial cell proliferation.

Methods: Posterior segments of bovine eyes were incubated with trypsin. After digestion of the tapetum, pieces of Bruch's membrane with the adherent choriocapillaris were scraped, digested again with a cocktail of enzymes, washed and cultured in MCDB 131 medium. Cells were incubated with 100 µg/ml thalidomide (dissolved in DMSO as recommended). Control cultures were incubated with DMSO alone or without any supplement. Cell proliferation after 24 hours was determined in a counting chamber. Purity of the cultures was controlled by immunohistochemical staining and labelling with low density lipoprotein.

Results: Thalidomide dissolved in DMSO inhibited proliferation of choriocapillary endothelial cells compared to control cultures incubated without any supplement. However, DMSO alone suppressed cell proliferation equally well.

Conclusion: In cultured bovine choriocapillary cells the inhibiting effect of thalidomide may at least in part be attributed to the solvent DMSO alone.

Choroidal neovascularisation (CNV) in age related macular degeneration is the leading cause of legal blindness in people aged 65 years and older. Laser treatment, photodynamic therapy, surgical interventions, and pharmacological approaches have been undertaken to find an adequate therapy. Since CNV represents neovascular growth from the choriocapillaris, anti-angiogenic drugs should be found that inhibit choriocapillary endothelial cell growth.

Thalidomide (α [N-phthalimido]-glutarimide) may have an inhibiting effect on new blood vessel growth. It is an odourless, white crystalline compound with low solubility in water. It is a derivative of glutamic acid with two rings and two optically active forms.¹

It has been suggested that thalidomide exerts its anti-angiogenic properties by generating toxic hydroxyl radicals.² In vivo it has been shown to have an anti-angiogenic effect against neovascular growth induced by vascular endothelial growth factor (VEGF) in the rabbit cornea,³ as well as against neovascularisation induced by basic fibroblast growth factor (bFGF) or VEGF in the mouse cornea.⁴ In vitro thalidomide has been shown to be effective in inhibiting human umbilical vein endothelial cell proliferation,⁵ as well as retinal pigment epithelial cell proliferation and migration.⁶

The aim of this study was to evaluate the effect of thalidomide on the proliferation of cultured bovine choriocapillary endothelial (CCE) cells.

MATERIALS AND METHODS

Isolation and culture of choroidal endothelial cells

Isolation and cultivation of choriocapillary endothelial cells was performed as described earlier (submitted for publication). In brief, bovine eyes were bisected and the anterior segment, vitreous, and retina were removed. The remaining eyecup was filled with Dulbecco's modified Eagle's medium (DMEM) containing 3% amphotericin B, 600 IU/ml penicillin, + 600 µg/ml streptomycin and was washed twice. Retinal pigment epithelium (RPE) cells were removed from Bruch's membrane by brushing with a small hairbrush, and the cup was filled again with trypsin (0.25%) ethylene diamine tetraacetic acid (EDTA) (0.02%) solution and incubated for 1 hour

at 37°C. The trypsin solution was removed and the cup was washed twice with DMEM containing 1% amphotericin B, 200 IU/ml penicillin, + 200 µg/ml streptomycin. Pieces of tapetum and Bruch's membrane with the adherent choriocapillaris were scraped off the choroid with a scalpel, and washed with at least 50 ml DMEM over nylon gauze (83 µm mesh). Tissue pieces macroscopically containing choroidal melanocytes were discarded. The remaining pieces were incubated in 10 ml of an enzyme solution (500 µg/ml collagenase, 200 µg/ml pronase, and 200 µg/ml DNAase I (Boehringer, Mannheim, Germany)) for 25 minutes at 37°C in a water bath. The resulting fragments were trapped again on a 83 µm nylon mesh and washed with 50 ml DMEM. They were then transferred into a conical tube with culture medium and centrifuged at 1200 rpm for 10 minutes. Cells from the middle layers were finally resuspended in MCDB-131 medium supplemented with 10 mM sodium bicarbonate, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, and 10% platelet-poor horse serum (all chemicals from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). CCE cells were seeded onto gelatin coated (Gelantine, 300 Bloom, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) 24 well tissue culture plates (Falcon, Heidelberg, Germany) and cultured in a humidified atmosphere of 5% CO₂ and 20% O₂ at 37°C. Culture medium was subsequently changed twice per week. Confluent cultures were released by weak digestion with trypsin-EDTA (0.1% + 0.02%) and spliced at a ratio of 1:3.

Immunohistochemical staining

A standard two stage indirect immunohistochemical technique was applied using anti-von Willebrand factor (vWF)/factor VIII antibody (clone F8/86, Dako A/S, Glostrup, Denmark), horseradish peroxidase conjugated species specific secondary antibody (Amersham, Braunschweig), and 3-amino-9-ethylcarbazole (ACE, Dako A/S, Glostrup, Denmark) as chromogen. Cells were counterstained with calcium red.

Labelling with low density lipoprotein

To test the incorporation of low density lipoprotein (LDL) acetylated and labelled with fluorescent

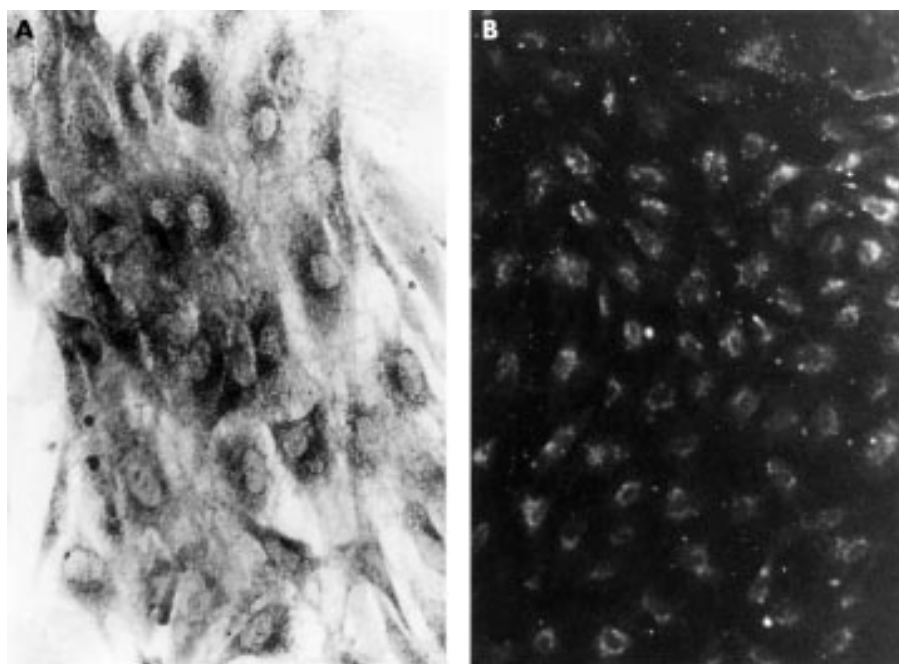


Figure 1 (A) Immunoperoxidase staining of CCE cells with anti-von Willebrand factor (vWF)/factor VIII antibody demonstrates positive, granular, perinuclear reaction product. (B) Labelling of CCE cells with Dil-Ac-LDL (micrograph by fluorescent microscopy). CCE cells show prominent phagocytosis of Dil-Ac-LDL.

1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL, TEBU, Frankfurt/Main, Germany), cell samples of each isolation and of each splicing procedure were grown on dishes (35 × 10 mm), and incubated for 4 hours at 37°C with CCE medium containing 10 µg/ml Dil-Ac-LDL. After incubation, the slides were washed with Dulbecco's phosphate buffered saline (DPBS) and covered with glycerol gelatin. The samples were examined with a Olympus IMT-2 fluorescence microscope.

Reagents

Thalidomide (Tocris, Biotrend, Cologne, Germany) was dissolved in DMSO at a concentration of 25 mM/ml according to the manufacturer's recommendation. A final concentration of 100 µg/ml thalidomide was used as recommended for cell culture.

Experimental design

CCE cells (0.6 ml cell suspension, 2×10^4 cells/ml) from passage 2–3 were plated onto gelatin coated 24 well tissue culture plates. After 24 hours of incubation, the medium was changed and non-confluent cells were further incubated for 24 hours with the appropriate medium containing 100 µg/ml thalidomide. Control cultures were incubated with DMSO alone (concentration equivalent to that used for 100 µg/ml thalidomide) or without any supplement. After removing the medium, cells were washed twice with DPBS and detached using 0.25 ml of a trypsin (0.1%) and EDTA (0.02%) solution and fixed in 0.25 ml paraformaldehyde (4% in DPBS). Cells were counted in a counting chamber.

Statistical analysis

All experiments were carried out in fourfold and performed at least four times. The results were expressed as the mean (SE) of all cultures. For the statistical analysis, Student's unpaired *t* test was used. A confidence level of $p \leq 0.05$ was considered statistically significant.

RESULTS

Cultures of isolated CCE cells were evident 6–10 days after the initial plating. Given a splicing ratio of 1:3, cultures normally

became confluent in about 8–10 days. Cultured CCE cells displayed a cobblestone phenotype and grew in confluent contact inhibiting monolayers. The cells stained with anti-von Willebrand factor (vWF)/factor VIII antibodies (Fig 1A) and incorporated Dil-Ac-LDL (Fig 1B), thus proving their nature as endothelial cells. Purity was over 90% in all cultures.

Effect of thalidomide on choriocapillary endothelial cell proliferation

Cell count 24 hours after incubation revealed a mean of 2.3 (SE 0.8) $\times 10^4$ cells/ml in wells incubated with 100 µg/ml thalidomide. Controls incubated without any supplement reached 3.8 (0.9) $\times 10^4$ cells/ml, and wells incubated with DMSO alone in a concentration equivalent to that needed to dissolve 100 µg thalidomide showed a mean of 2.4 (0.8) $\times 10^4$ cells/ml (Fig 2). There was no statistically significant difference between cell count of samples treated with 100 µg/ml thalidomide and those treated with DMSO (in the same concentration) alone ($p = 0.74$). Compared to the controls cell count was statistically significantly reduced in both the thalidomide ($p < 0.001$) and the DMSO ($p < 0.001$) treated samples.

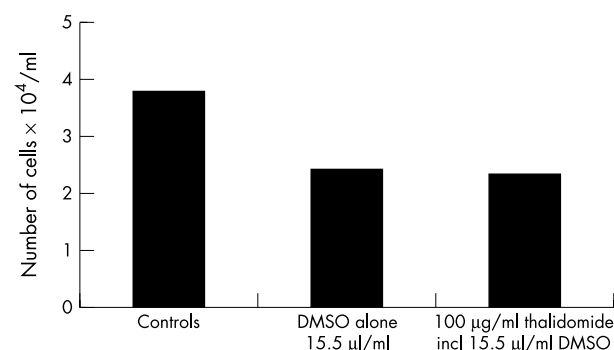


Figure 2 CCE cell count 24 hours after incubation with 100 µg/ml thalidomide, DMSO (in a concentration equivalent to solve 100 µg/ml thalidomide), and culture media without supplement (control).

DISCUSSION

The initial aim of this study was to test the capability of thalidomide to suppress choriocapillary endothelial cell growth.

Choroidal neovascularisation is still a challenge to treat. By establishing choriocapillary cell cultures, potential pharmacological therapies can be tested before going on to animal studies. With the method described we were able to obtain over 90% purity in all cultures. Therefore, the procedures performed proved to be an excellent technique for the creation of choriocapillary endothelial cell cultures for in vitro experiments with drugs such as thalidomide.

Thalidomide in its present form is not soluble in water. Therefore, for in vitro experiments, DMSO is commonly recommended for use as a solvent. Using 25 mM thalidomide per ml DMSO, the solution approximately achieves saturation (stock solution). Therefore, a reduction in the amount of DMSO might not be possible.

Our results show an inhibition of choriocapillary endothelial cell proliferation using 100 µg/ml thalidomide dissolved in DMSO compared to the controls without supplement. However, we demonstrated that DMSO alone in a concentration equivalent to dissolve 100 µg thalidomide could suppress cell proliferation equally well (Fig 2).

Only a few study groups have used thalidomide in in vitro experiments. Spraul and coworkers⁶ and Kaven and coworkers⁷ showed a statistically significant inhibitory effect of thalidomide dissolved in DMSO on migration and proliferation of RPE cells compared to controls without any supplement. However, they did not run a control with DMSO alone. Our findings are in contrast with those of Moreira *et al.*,⁵ who demonstrated a dose dependent inhibition of human umbilical vein endothelial cell proliferation by thalidomide in DMSO compared to samples treated with DMSO alone, which showed no inhibition. However, they found no effect of either thalidomide or DMSO on proliferation of human glioma cells. Bauer and coworkers⁸ demonstrated an inhibiting effect of thalidomide on microvessel formation of rat aortas and on human aortic endothelial cell proliferation, but only in the presence of human or rabbit microsomes. In their study, controls were exposed to DMSO alone and showed no inhibition.

To date, the mechanism of a possible anti-angiogenic effect of thalidomide is still unclear.¹⁻⁹ It might be species specific⁸ and caused by the action of one of its metabolites.⁸ The same may be true for DMSO. This may explain the difference

between our findings on bovine cells and those of Moreira *et al.*⁵ on humans and Bauer *et al.* on rats and humans.⁸ Recently, efforts have been made to synthesise water soluble thalidomide prodrugs.⁹ Using those prodrugs for in vitro experiments will make DMSO superfluous and the results of cell culture experiments with thalidomide-like substances easier to interpret.

In conclusion, we found an inhibitory effect of thalidomide dissolved in DMSO on choriocapillary endothelial cell proliferation. However, this effect may at least in part be attributed to the solvent DMSO alone. Therefore, all in vitro experiments using thalidomide without running a control trial with the solvent alone have to be interpreted with the utmost caution.

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