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Biological response of endothelial cells and its modulation by cytokines: prospects for therapy and bioprocesses

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Abstract

Endothelial cells are involved in important pathological situations. They could be the target for infectious processes as for example in Cowdriosis, an important disease in cattle due to the rickettsia *Cowdria ruminantium* prevalent in the south of the Sahara. They are also connected to angiogenic processes related to tumor invasion.

Our results indicate that AIDS related Kaposi sarcoma cells may be of endothelial origin. We conclude from our data that the mobility of those cells, related to the expression of the metalloproteinases (especially the 92 kD form of the enzyme), is an important factor in Kaposi sarcoma dissemination and is the main factor limiting the scale up of Cowdriosis vaccine production in Bovine Umbilical Endothelial Cell line. We showed that PMA and TNF increased the 92 kD Metalloproteinase and that TGF β , produced in an inactive form in cultures of Human Umbilical Vein Endothelial Cells, is a potential inhibitor of Kaposi sarcoma spreading, and could also be useful in improving our process for *Cowdria ruminantium* vaccine production, since it reduces the sensitivity of the cells to mechanical stress without affecting significantly the overall infectious process.

Abbreviations: ACM, Activated Conditioned Medium; CM, Conditioned Medium; NM, Normal Medium; BUEC, Bovine Umbilical Endothelial Cells; HUVEC, Human Umbilical Vein Endothelial Cells; KS, Kaposi sarcoma; MMPases, Metalloproteinases; PMA, Phorbol Myristate Acetate; TGF, Tumor Growth Factor; TNF, Tumor Necrosis Factor

Introduction

Endothelial cells play a crucial role in a number of pathological conditions and in their regulation. Blood coagulation, inflammation, immune response, infections and tumoral invasion processes are of course of major concern in this respect. Angiogenesis mechanisms are particularly relevant to the understanding of these different situations (Fajardo, 1989; Jaffe, 1987; Folkman and Shing, 1992). Undoubtly also, cytokines play an important role in the regulation of pathophysiological responses of endothelial cells and in their overall behaviour.

We have been interested in two important pathological situations for which better knowledge of endothelial properties could permit the identification of therapeutic solutions:

1. The first situation concerns a deadly cattle disease (Cowdriosis or Heartwater) prevalent in the south of the Sahara and which is caused by the multiplication of an obligate intracellular bacteria, the rickettsia *Cowdria ruminantium*, for which the final cellular targets are endothelial cells from the brain capillary vessels (Totté, Jongejan *et al.*, 1994).
2. The second situation concerns Kaposi (KS), an angioproliferative disease prevalent in AIDS patients

and characterized by the dissemination of vascular lesions on the skin or mucosal tissues. (Blankaert *et al.*, 1994; Simonart *et al.*, 1996).

Migration and proliferation of endothelial cells, being relevant to the understanding of both conditions, we have first studied the effect of components of the cytokine network on the expression of the matrix metalloproteinases (MMPases), since these enzymatic activities are related to vascular permeability and cellular mobility (Coulombe *et al.*, 1989). We have used in this study therefore, endothelial cells of bovine origin routinely used to cultivate *Cowdria ruminantium* (a bovine umbilical endothelial cell line – BUEC) and of human origin, primarily the human spindle like cells we have isolated from AIDS related-KS biopsies, comparing them to primary cultures of human umbilical vein endothelial cells (HUVEC) and to SV40 T antigen transfected HUVEC (pSC1 cells) or to 1/2 C10 (HUVEC-Human Osteosarcoma cell line hybrid).

The two main related questions addressed here were the following:

- Could we find conditions enabling *in vitro* maintenance of spindle like cells obtained from Kaposi sarcoma that we characterized here as of endothelial origin, in order to select potential treatment (including using cytokines) to reduce growth and invasiveness of the KS cells?
- Could we find conditions permitting maintenance of endothelial cell cultures in spinner bioreactors for the extended period of time (more than two weeks) necessary to produce enough of *Cowdria ruminantium* variants to be tested as potential attenuated vaccine which was up to now unavailable?

The approach we took to answer these questions was to identify and characterize factors which, while inhibiting cell mobility related to the invasive process, would be a potential inhibitor for KS spreading, and would, by increasing their adhesion on microcarriers, limit their sensitivity of mechanical stress during maintenance in bioreactor for *Cowdria ruminantium* production.

Material and methods

Cells, culture conditions and procedure for infection with Cowdria ruminantium

Routinely the cells were passaged by trypsinization with 0.05% trypsin and 0.02% EDTA in PBS. Cell number was determined in triplicate by Trypan blue

exclusion with an hemacytometer. Infection with *Cowdria ruminantium*: the Senegal stock of the rickettsia used was originally obtained from Dr F. Jongejan from Utrecht University, and infection in endothelial cells was operated and revealed as previously described (Totté, Blankaert *et al.*, 1993).

HUVEC cells. (Primary human umbilical vein endothelial cells) were isolated from full-term umbilical cord vein treated as described by Gimborne *et al.* (1974). Cells isolated by collagenase treatment of 7 umbilical cords were pooled and grown on gelatin-coated disks in Minimum Essential Medium containing D-Val, with 20% decompemented foetal calf serum, 100 µg/ml endothelial growth factor, and Penicillin (100 U/ml) and streptomycin (100 µg/ml). Incubation was operated at 37 °C in humid atmosphere at 5% CO₂/95% O₂.

Kaposi sarcoma cells. KS cell cultures were established as previously described (Blankaert *et al.*, 1994), and as it will be reported more extensively elsewhere. In brief, biopsies of KS lesions minced into small pieces were treated for 90 minutes at 37 °C with Trypsin (0.3% –1% EDTA) or collagenase (1 mg/ml from *Clostridium histolyticum*). After centrifugation the cells were washed and cultivated in the same medium as used for HUVEC and grown similarly with 20% decompemented foetal calf serum and 1% non essential amino acids.

pSV1 cells. HUVEC cells transfected with T antigen from SV40 (Lassale *et al.*, 1992) were kindly supplied to us by Dr V. Fafeur from Pasteur Institute (Lille). Those cells were grown in RPMI 1640 medium with 10% Foetal calf serum and the usual proportion of antibiotics.

1/2 C10 cells. HUVEC – Human Osteosarcoma cell line was provided by the group of Prof. Kattinger (Vienna, Austria).

BUEC cells. (Bovine Umbilical endothelial cells) were originally obtained from Dr F. Jongejan from Utrecht University, The Netherlands. They were grown in DMEM (GIBCO Life Technologies) in presence of 10% decompemented Foetal Calf Serum, and the usual amount of antibiotics, 1% essential amino acids and 1% glutamine.

Microcarriers used. Routinely Cytodex 1, 2 and 3 are used, but in some experiments Cytocell or Cultispher S microcarriers have been tested also. The microcarriers obtained in a dry state were treated in the conditions recommended by the producer and routinely washed twice with prewarmed culture medium before use. Spinner cultures are made in CO₂ incubators under agitation of 30 rpm.

Conditioned medium and its "activation". Cultures of HUVEC in tissue culture flasks of 80 cm² were maintained for 48 h in fresh medium which was then harvested and kept as "conditioned medium" (CM). Its activation resulted from acidification until pH2 with 10 M HCl for 1 h at 4 °C, after which neutralization was quickly operated with 10 M NaOH.

Cytokines and other factors used for cell treatment

TNF α . Human recombinant Tumor Necrosis Factor (TNF α). TNF at 10⁸ cytotoxic units/mg was received from Dr M. Bochaus (Hoffman LaRoche, Basel, Switzerland).

PMA. 4 Beta-Phorbol-12-Myristate-13 Acetate (PMA) was from Sigma (St Louis; Mo, USA).

TGF β . Human natural TGF β obtained from Promega is diluted at 30 μ g/ml in normal medium without serum for investigating its effect between 0.25 and 1 ng/ml. During treatment, medium was changed every day and collected after 24 and 48 h incubation without serum before testing MMPases by Zymography.

Antibodies against TGF β . Dehydrated Monoclonals against TGF β 1, 2 and 3 at 300 pg TGF β or rabbit polyclonals used at a dilution of 1/200 were also from Promega.

Gelatin zymography

The gel electrophoresis analysis of Metalloproteinases (MMPases) were performed by the method of Heusen and Dowdle (1980) adapted as previously described (Marique *et al.*, 1991). 10% gel electrophoresis was operated at 4°C at 20 mA for 3 hours. After soaking in 2.5% Triton X100 to remove SDS, gels were incubated overnight in a buffer containing 5 mM CaCl₂, 0.02% Sodium Azide, and 50 mM Tris pH 7.5. Gels were then stained in 0.5% Coomassie brilliant blue in acetic acid-isopropanol-water (1:3:6) and photographed on a light-box. The analysis of conditioned medium requires to

incubate cells without serum for at least 24–48 h before testing since serum contains MMPases.

Immunofluorescence techniques

Cells (HUVEC, BUEC or Spindle like cells) were treated first with Factor VIII mouse antibodies (1/150) and second by fluorescein conjugated immunoglobulins (DAKO).

Results

Expression of factor VIII in the cells used

All the cells used in the present study – BUEC, HUVEC, KS (Figure 1A, B, C) and 1/2 C 10 (Figure 1D) and psVI (data not shown) expressed Factor VIII when tested with mouse antibodies detected by fluorescein conjugated immunoglobulins. The expression is rather intense and in fact less diffuse in BUEC and KS cells, than in HUVEC and in the derived 1/2 C cells. This indicated that, at least at the early passage used here, KS cells were originating from endothelial related tissues. Knowing the relative difficulties to grow endothelial cells *in vitro*, and in order to establish conditions that could hopefully stabilize their maintenance in culture, we have tested the effect of cytokines and other factors from which we could expect a positive effect due to their role in the expression of Metalloproteinases (MMPases) involved in the mobility and invasiveness of endothelial cells.

Effect of PMA and TNF on MMPases expression in BUEC cells

Zymography revealed in BUEC cells (Figure 2A and B) the activity of different MMPases. We showed here that the relative expression of the 92 kD MMPase relative to the 72 kD enzyme, is much more increased by PMA. (Maximal activity is observed at 10 nM). TNF also increased relatively more efficiently the expression of the 92 kD MMPase versus the 72kD enzyme (Optimal activity is obtained between 5 and 10 ng/ml).

The same approach has been taken to analyze MMPases expression in KS cells (Figure 3A and B), and the same conclusions can be drawn. PMA increases relatively 92 kD MMPase (maximal activity reached at 8 nM) – and TNF also. (The activity is clearly detected at 5 ng/ml).

Figure 1. Expression of Factor VIII in cells in culture. A: BUEC – B: HUVEC – C: 1/2 C10 – D: KS cells.

Figure 2. Effect of PMA and TNF on MMPases expression in BUEC. A: Analysis by Zymography of the effect of PMA (from left to right: concentration of 10 – 5 – 1 – 0.5 – 0.1 and 0 nM) on the level of 92 kD and 72 kD MMPases activity in conditioned medium. B: Analysis by zymography of the effect of TNF (from left to right: 10 – 5 – 3 – 1 – 0.5 – 0.1 and 0 ng/ml).

As it is known that the level of expression of the 92 kD MMPase is related to cell mobility, as observed for example in metastatic processes, zymography was used to select conditions in which cell movement may be potentially reduced.

Effect of conditioned medium and TGF β on MMPases expression in BUEC and KS cells, and on their growth in vitro

Zymographic analysis of the expression by BUEC or KS cells under conditioned medium treatment (respectively Figure 4A and Figure 5A) or TGF β action (Fig-

ure 4B and 5B) revealed that 92 kD MMPase expression is clearly reduced by both treatments. In parallel experiments (data not shown) we have observed that TGF β , in a dose dependent manner, reduced BUEC cell growth. More than 40% inhibition was obtained at 1 ng/ml, a similar effect being observed with conditioned medium. We tested, therefore using monoclonal antibodies against TGF β , if the effect obtained with conditioned medium on the growth of the endothelial cells is due to this factor. Our results showed (Figure 6) that it is indeed the case both in BUEC and KS cells grown in Petri dishes. Conditioned medium, before acid activation already caused a partial inhibi-

Figure 3. Effect of PMA and TNF on MMPases expression in KS cells. A: Analysis by zymography of the effect of PMA (from left to right at 20, 16, 8, 2 and 0 nM). B: Analysis by zymography of the effect of TNF (left 5 ng/ml – right: 0 ng/ml).

Figure 4. Effect of conditioned medium and TGF β on MMPases expression in BUEC. A: Effect of activated conditioned medium: Analysis by zymography of the effect on the expression of 92 kD MMPase (left: control – right: conditioned medium). B: Effect of TGF β : Analysis by zymography of the effect on the expression of 92 kD MMPase (from left to right: NM/normal medium – CM/conditioned medium – ACM/Activated Conditioned Medium – NM + TGF β 1 ng/ml).

tion. The effect is totally reversed by a treatment with Anti TGF β antibodies. Partial activation of preTGF β (or a closely related factor) is therefore the cause of the

Figure 5. A: Effect of ACM in KS cells: Analysis by zymography of the effect of activated conditioned medium (right: control conditioned medium – left: activated conditioned medium) on the expression of MMPases. B: Effect of TGF β in KS cells: Analysis by zymography of the effect of TGF β (from left to right: 0 – 5 – 4 – 3 – 2 and 1 ng/ml) on the expression of 92 kD MMPase.

Figure 6. Inhibition of growth of BUEC and KS cells by conditioned medium and its reversal by anti TGF β . Growth of BUEC and KS cells is followed in Petri dishes for 48 h (the time necessary to reach confluence in normal medium) and cells are counted in control cultures and in cultures with CM, ACM and ACM + anti TGF β antibodies (dose neutralizing 1 ng/ml).

Figure 7. Comparison of the spreading of BUEC on Cytodex 2 and 3. NM is used in both cases.

effect. This indicates that TGF β could be of interest to reduce KS lesions spreading *in vivo*.

Figure 8. Inhibition of colonization of Cytodex 3 by BUEC and KS cells in conditioned medium. BUEC and KS cells were seeded on Cytodex 3 on confluent monolayers and counted after 48 hours.

Figure 9. Maintenance of BUEC in "Spinner flasks". Cytodex 3 seeded on BUEC confluent monolayers up to saturation were incubated in normal medium (NM) and in activated conditioned medium (ACM) in "spinners" flasks. Cells on microcarriers were counted daily for over a week.

Figure 10. Infection of BUEC by *Cowdria ruminantium* in presence and absence of TGF β . Cell nucleus is dark stained and vacuoles where *Cowdria ruminantium* develop are indicated by arrows. Staining is operated 9 days after infection or mock infection. A: uninfected control – B: Infected control – C: infected in presence of 1 ng/ml TGF β .

Colonization of microcarriers by BUEC and KS cells and its inhibition by activated conditioned medium

We compared different microcarriers (Cytodex 1, 2 and 3, Cytocell, and Cultispher S were used). Cytodex were more efficiently colonized than Cytocell and Cultispher (data not shown) and Figure 7 indicates that BUEC cells spread better on Cytodex 3 than on Cytodex 2, after seeding them on a confluent monolayer culture. Therefore, Cytodex 3 was selected as microcarrier for subsequent studies. Using Cytodex 3, we showed that in 48 hours, the microcarriers have been invaded by KS or BUEC cells (up to 30-35% saturation) when seeded on a confluent monolayer, while conditioned medium, especially after activation, is readily inhibiting the colonization (Figure 8). Therefore cell motility is inhibited in parallel to the reduction of the expression of the 92 kD MMPase.

Maintenance of BUEC cells in "Spinner" flasks

In order to produce in mass the *Cowdria ruminantium* stock that we intend to test as an attenuated vaccine, we followed first the behavior of Cytodex 3 microcar-

riers colonized by BUEC cells in "spinner" flasks in two conditions – with normal medium or with activated conditioned medium. We observed (Figure 9) that cells seeded on confluent BUEC monolayers, when incubated in normal culture medium, detached readily from the microcarriers, and after a week of incubation, all cells died. On the contrary, when incubated with activated conditioned medium, most of the cells were maintained in healthy conditions for the long period of time required for *Cowdria ruminantium* development.

We therefore checked if *Cowdria ruminantium* production could be operated in presence of TGF β .

Infection of BUEC cells by *Cowdria ruminantium* in presence of TGF β

Cowdria ruminantium develop normally in BUEC cells in the presence of TGF β (1 ng/ml) as shown in Figure 10. Vacuoles which are hosting *Cowdria ruminantium* are formed similarly in the presence or absence of TGF β after the 9 days necessary for the main part of the rickettsia maturation process. Appropriate conditions for an efficient vaccine production can therefore be now foreseen.

Discussion

Given our double goal in this study to characterize Kaposi spindle like cells in order to check potential inhibitors for treatment of the corresponding disease, and to define maintenance conditions for *Cowdria ruminantium* infected BUEC cells in order to propose an appropriate vaccine production process – the present approach has been quite rewarding.

Kaposi Sarcoma (KS) cells that we isolated from AIDS patients biopsies, expressed Factor VIII, indicating their endothelial origin, and as BUEC, expressed 92 kD MMPase at high level when stimulated either by PMA, TNF, or by both agents as indicated previously (Blankaert *et al.*, 1994). We showed here that TGF β and activated conditioned medium reduced the expression of 92 kD MMPase, appearing therefore as a potential cell mobility inhibitor of interest for KS treatment. It appeared also for the same reason as a valuable maintenance factor for Cowdriosis vaccine production. Therefore, the scale up of endothelial cell production process, recently proposed by S. Duvar *et al.* (1996), could be adapted for infection with *Cowdria ruminantium*. (Totté, Blankaert *et al.*, 1994).

The first part of our conclusions should now be further investigated by appropriate *in vivo* tests before clinical studies, while the second part is currently under investigation in our laboratory, in parallel to a related metabolic engineering approach for which we have briefly described preliminary conditions (C. Alloin *et al.*, 1996).

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