Cyclooxygenase-2 activity is necessary for the angiogenic properties of oncostatin M

Jérôme Pourtau[a], Farrokh Mirshahi[a], Hong Li[a], Marc Muraine[a,b], Loïc Vincent[a], Alain Tedgui[c], Jean-Pierre Vannier[a], Jeannette Soria[d], Marc Vasse[a], Claudine Soria[a,e,*]

[a] *Laboratoire DIFEMA, UFR de Médecine et Pharmacie de Rouen, Boulevard Gambetta, 76183 Rouen, Cedex, France*
[b] *Service d'Ophtalmologie, Hôpital Ch. Nicolle, Rouen, France*
[c] *INSERM U141 Hôpital Lariboisière, Paris, France*
[d] *Laboratoires de Biochimie et Sainte Marie (J.S.), Hôtel-Dieu, Paris, France*
[e] *INSERM U353, Hôpital Saint Louis, Paris, France*

Received 23 June 1999

Abstract Macrophages play a major role in angiogenesis. We recently reported that oncostatin M (OSM), a cytokine of the interleukin (IL)-6 family secreted by macrophages, has a potent angiogenic activity on human microvascular endothelial cells (HMEC-1), but has no effect on macrovascular cells (human umbilical vein endothelial cells (HUVECs)). In this work, we show that in HMEC-1, OSM (0.5–2.5 ng/ml), leukemia inhibitory factor (LIF) (25 ng/ml), bFGF (25 ng/ml) and IL-1β (5 ng/ml) induced production of cyclooxygenase (COX)-2. In contrast, in HUVECs, neither OSM nor LIF induced COX-2 mRNA, suggesting that COX-2 might be implicated in the angiogenic activity of OSM. This was confirmed by the inhibiting effect on OSM-induced HMEC-1 proliferation of specific COX-2 inhibitors. In vivo studies confirmed this findings. We conclude that induction of COX-2 by OSM is necessary for its angiogenic activity, but is not sufficient since IL-1β, which also induces COX-2 in HMEC-1, has only a poor proliferative effect.

Key words: Oncostatin M; Angiogenesis; Cyclooxygenase-2; Monocyte; Macrophage

1. Introduction

Macrophages are prominent cellular components of atherosclerotic plaques. Cytokines secreted by activated macrophages are involved in the pathogenesis of atherosclerotic plaque development and plaque rupture, which is responsible for the acute ischemic syndrome. Among these cytokines, we have previously suggested that oncostatin M (OSM), a cytokine produced by activated monocytes/macrophages, could play a major role in the development and complication of atherosclerosis [1]. This may explain the results of epidemiologic studies showing that fibrinogen is an important marker of cardiovascular disease. This deterious role of OSM is supported by the observation that OSM was identified in the atherosclerotic plaque [2].

Because the neovasculature of the plaque contributes to its fragilization, we were interested in analyzing the effect of OSM on angiogenesis. We have previously shown that OSM, and to a lesser extent leukemia inhibitory factor (LIF), induces a potent proliferative effect on microvascular cells (human microvascular endothelial cells (HMEC-1)) and a strong angiogenic activity both in in vitro and in vivo models. Interestingly, the effect of OSM was limited to microvascular cells, since OSM did not induce any proliferative effect in endothelial cells (ECs) from the human macrovasculature (human umbilical vein endothelial cells (HUVECs)) [3]. The mechanisms responsible for this angiogenic effect have not been elucidated. The effect seems to be direct, as other known angiogenic factors were not significantly increased by OSM.

Cyclooxygenase (COX, prostaglandin (PG)-endoperoxide synthase) converts arachidonic acid to PG H_2, which is then metabolized by other enzymes to various PGs, including prostacyclin and thromboxane. COX exists in, at least, two isoforms with a similar molecular weight (70–72 kDa). COX-1 is expressed constitutively in various tissues. In contrast, the expression of COX-2 is induced or up-regulated by mitogens, cytokines or tumor promoters [4].

Since COX-2 has been implicated in angiogenesis [5,6], we analyzed in this work the expression of COX-2 by ECs and its possible contribution to OSM-induced angiogenic activity. To that end, we investigated the effect of OSM on COX-2 expression in ECs (HMEC-1 and HUVECs). We also studied the effect of COX inhibitors on microvascular EC proliferation and migration in vitro and on OSM-induced angiogenesis in the in vivo rabbit cornea model [7].

2. Material and methods

2.1. Cytokines

Recombinant human OSM, LIF, bFGF and interleukin (IL)-1β were supplied from R&D Systems (Minneapolis, MN, USA). Recombinant human IL-6 was a generous gift of Sandoz (Basel, Switzerland). Antibody against COX-2, indomethacin, ibuprofen and NS-398 were obtained from TEBU (Le Perray-en-Yvelines, France).

2.2. Cell culture

The HMEC-1 cell line was provided by Dr Ades (Center for Disease Control and Prevention, Atlanta, GA, USA), who established this cell line by transfecting human dermal ECs with SV40 A gene product and large T-antigen [8]. HMEC-1 cells were cultured in MCDB131 medium (Sigma, St. Louis, MO, USA) supplemented with 15% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml epidermal growth factor (EGF) (Euromedex, Souffelweyersheim, France) and 1 mg/ml hydrocortisone (Pharmacia-Upjohn, Saint Quentin en Yvelines, France).

HUVECs, collected as described by Jaffe et al. [9], were cultured under similar conditions as HMEC-1 but the percentage of FCS was lower (8%).

*Corresponding author. Fax: (33) (2) 35 14 83 40.

Abbreviations: OSM, oncostatin M; LIF, leukemia inhibitory factor; IL, interleukin; COX, cyclooxygenase; EC, endothelial cell

0014-5793/99/$20.00
PII: S0014-5793(99)01301-0

2.3. Cell proliferation assay

For the proliferation assay, EGF was omitted and we used the minimal concentration of FCS allowing for sufficient viability of the cells (7.5%). Briefly, after trypsinization, the cells were seeded at a concentration of 50×10^3 cells per well in a 24-well plate (Nunc, Roskilde, Denmark) and incubated with the cytokines. The medium was changed every 2 days and the cell number was measured at day 5 by counting the cells in a particle counter (Coulter Z1, Coultronics, Margency, France) after detachment by trypsin (0.05%, w/v, Sigma).

2.4. Chemotaxis assay

HMEC-1 cells were detached with EDTA 0.5 mM, washed twice in phosphate-buffered saline (PBS) and resuspended in MCDB131 with 0.2 mg/ml bovine serum albumin (BSA, Sigma). Fifty thousand cells were seeded in the upper chamber of a Transwell insert (PTFE membrane with 8 μm diameter pores, Dutcher, Brumath, France). The lower chamber was filled with 1 ml of MCDB131 with 2 mg/ml BSA and the cytokine to be tested. In order to test the effect of a COX-2 inhibitor on cytokine-induced chemotaxis, NS-398 was added to the upper chamber at a final concentration of 50 μM. After 24 h, migrated cells were scraped from the lower surface of the membrane with a cell scraper (Nunc) and then suspended in the medium of the lower chamber to count all migrating cells (both adherent and cells in suspension). These cells were counted with a hemocytometer (Coulter Z1, Coultronics).

2.5. Total RNA extraction

Cells were incubated in a 6-well plate (Nunc) until confluent and then incubated for 3 or 24 h with the cytokines. Cells were then detached with 0.05% trypsin and washed twice in Dulbecco's PBS. Total RNA extraction was performed using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Oligonucleotide primers (which flank intron DNA) HSCOX2-S (5′-CCGGACAGGATTCTATGGAGA-3′ sense bases 232–252) and HSCOX2-AS (5′-CAATCATCAGGCACAGGAGG-3′ antisense bases 531–512) were used in a RT-PCR assay. For each reaction (volume of 50 μl), the mixture containing 0.4 pg of total RNA, 50 pmol of each primer, 0.2 μM dNTPs, 1 mM $MgSO_4$, 5 μl of 10X AMV/Tfl reaction buffer was prepared and heated for 2 min at 94°C. When the temperature was decreased to 48°C, 2 U AMV and 2 U Tfl enzymes were added to the reaction mixtures. The reverse transcription was performed at 48°C for 50 min. The reaction products were then subjected to 30 cycles of PCR: 94°C for 2 min, 60°C for 30 s and 68°C for 1 min, followed by 29 cycles of 94°C for 1 min, 60°C for 30 s and 68°C for 1 min and finally, 68°C for 7 min.

RT-PCR amplification of β-actin (5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ sense primer; 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′ antisense primer) was performed as control under the same conditions as above. The COX-2 (300 bp) and the β-actin (838 bp) mRNA amplification products were analyzed in agarose gel (1.5%) electrophoresis using ethidium bromide staining.

2.7. Western blot analysis

Cells cultured in six well plates were incubated with IL-1β (5 ng/ml) or OSM (0.5, 1 and 2.5 ng/ml) for 24 h. Cells were washed in PBS, trypsinized and resuspended in lysis buffer RIPA (10 mM Tris-HCl, 1% sodium desoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), pH 7.5) containing one tablet per ml protease inhibitor mixture (Boehringer Mannheim, Roche diagnostic, Meylan, France) for 30 min on ice. Cell lysate was collected by centrifugation at 12 000 rpm at 4°C and the amount of protein was determined by the method of Bradford [10].

Eighty pg of protein per lane for each sample was run in a 10% SDS-polyacrylamide gel before transfer onto a nitrocellulose membrane (Amersham, Courtaboeuf, France). The mnembrane was incubated in blocking buffer (10 mM Tris-HCl, 100 mM NaCl, 0.05% Tween-20 and 5% fat-free milk, pH 7.5) for 2 h, then incubated for l h in a goat IgG against human COX-2 (TEBU) at a final concentration of 0.5 pg/ml. The membrane was washed three times in Tris-Tween-20 buffer and incubated for another hour with horseradish peroxidase-conjugated rabbit anti-goat IgG serum (Biosys, Compiegne, France). After washing, COX-2 was detected using a Luminol ECL detecting kit (Amersham).

2.8. In vivo angiogenesis assay

Adult New-Zealand white rabbits (Charles River, St. Aubin-les-Elbeuf, France) weighing 1.5–2 kg were anesthetized with ketamine, (Ketalar 25 mg/kg intraperitoneally, Parke Davis, Courbevoie, France). The eyes were topically anesthetized with oxybuprocaine (Cibésine, Laboratoire Chauvin, France) and the globes were proptosed with jeweler forceps. Using an operating microscope, a central, intrastromal linear keratotomy was performed with a surgical blade. A lamellar micropocket was then dissected and was extended to within 2 mm of the superior limbus. Beforehand, dehydrated (1×2 mm) pellets made of 70% hydratable hydrogel were imbibed with vehicle only (sterile water, Eurobio) or 100 ng/implant of OSM and placed into the preformed corneal pocket. To test the effect of a COX inhibitor on OSM-induced angiogenesis, rabbit eyes received two drops of flurbiprofen 0.03% (Ocufen, Allergan, Mougins, France) three times a day during the period of the test.

The eyes were routinely examined by slit lamp biomicroscopy on postoperative day 8 after implantation. Rabbits were anesthetized with ketamine and the eyes were proptosed. Photographs were made and the area of neovascularization was evaluated and scored: 0 = absence of neovascularization, 1 = area of neovascularization <2 mm^2, 2 = area of neovascularization between 2 and 4 mm^2, 3 = area of neovascularization ≥4 mm^2. The studies have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals. In order to analyze whether the effect of cytokines on angiogenesis is direct or due to infiltration by inflammatory cells, histological studies were performed on sections after hematoxylin-eosin staining.

2.9. Statistical analysis

Significant values were determined using a two-tailed non-parametric Mann-Whitney test using the InStat software (Sigma). The results are expressed as mean value ± S.E.M. $P<0.05$ was considered as significant.

3. Results

3.1. Induction of COX-2 mRNA in HMEC-1 by OSM

The effect of OSM was first studied on COX-2 mRNA levels. Cells were incubated with increasing concentrations of OSM or 5 ng/ml of IL-1β, which is known to be a potent inducer of expression of COX-2 mRNA in various cell types. A concentration-dependent increase in COX-2 mRNA expres-

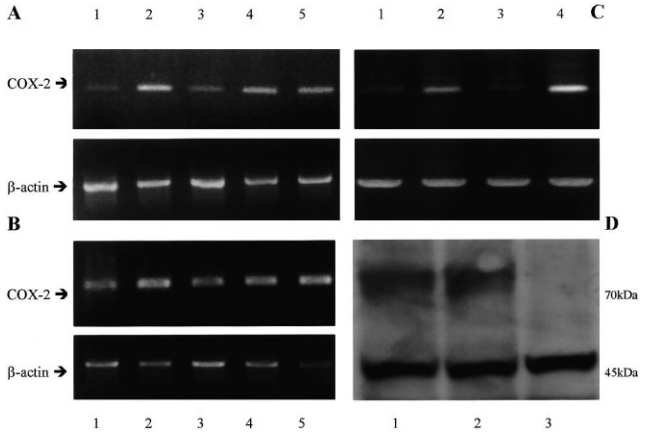

Fig. 1. Effects of OSM, IL-6, LIF or IL-1β on COX-2 mRNA and protein expression. (A) COX-2 and β-actin mRNA expression after a 3 h incubation. 1: control, 2: IL-1β 5 ng/ml, 3: OSM 0.5 ng/ml, 4: OSM 1 ng/ml, 5: OSM 2.5 ng/ml. (B) COX-2 and β-actin mRNA expression after a 24 h incubation. 1: control, 2: IL-1β 5 ng/ml, 3: OSM 0.5 ng/ml, 4: OSM 1 ng/ml, 5: OSM 2.5 ng/ml. (C) COX-2 and β-actin mRNA expression after a 3 h incubation. 1: control, 2: LIF 25 ng/ml, 3: IL-6 10 ng/ml, 4: bFGF 25 ng/ml. (D) Western blot of COX-2 protein expression in HMEC-1. 1: OSM 2.5 ng/ml, 2: IL-1β 5 ng/ml, 3: control.

sion in response to OSM occurred after 3 h (Fig. 1A). However, IL-1β induces a more potent increase. The fact that a RT-PCR assay reveals COX-2 mRNA in control HMEC-1 could be due to the culture conditions. After a 24 h incubation, Fig. 1B shows a persistent concentration-dependent increase in COX-2 mRNA expression. LIF (25 ng/ml) and bFGF (25 ng/ml) also induced an increase in COX-2 mRNA expression after a 3 h incubation, while IL-6 did not modify COX-2 mRNA expression in HMEC-1 (Fig. 1C).

3.2. Western Blot analysis of COX-2 in HMEC-1 incubated with OSM

COX-2 protein was not detected in the absence of cytokine. However, when the cells were incubated for 24 h with IL-1β or OSM, COX-2 expression was stimulated. A band of 70 kDa was detected using specific anti-COX-2 antibodies (Fig. 1D). A protein of approximately 45 kDa was also detected but its expression did not change with treatment. This band was also observed by Bishop-Bailey et al. in vascular smooth muscle cells using another polyclonal antibody [11].

3.3. Induction of COX-2 mRNA in HUVECs

As OSM induces a significant increase in COX-2 mRNA expression in HMEC-1, we investigated whether such an increase could also be observed in macrovascular ECs. Results in Fig. 2 show that 25 ng/ml bFGF and 5 ng/ml IL-1β induced a net increase in COX-2 mRNA expression while OSM (2.5 ng/ml) only induced a poor increase and LIF (25 ng/ml) or IL-6 (10 ng/ml) were ineffective.

3.4. Non-steroidal anti-inflammatory drugs inhibit OSM-induced proliferation of HMEC-1

The effects of COX inhibitors were evaluated on HMEC-1 proliferation induced by 2.5 ng/ml OSM.

Aspirin incubated for 5 days at concentrations ranging from 55 μM to 2.78 mM (which did not modify the culture medium pH) induced a decrease in cell proliferation in the absence or in the presence of OSM (data not shown). This decrease in the number of cells compared with the control is due to a loss of cell viability as observed by Trypan blue incorporation.

We then used ibuprofen, which is known to inhibit both COX-1 and COX-2 and NS-398, a specific inhibitor of COX-2.

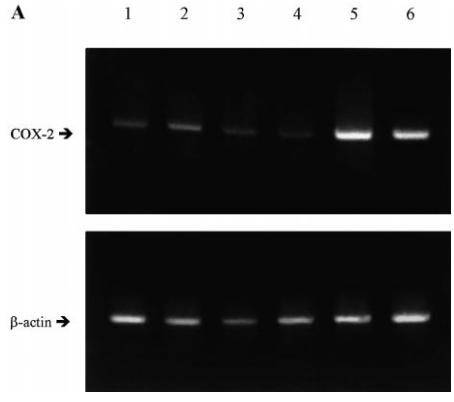

Fig. 2. Effects of 3 h incubation of HUVECs with OSM, LIF, IL-6, bFGF or IL-1β on COX-2 mRNA expression. 1: control, 2: OSM 2.5 ng/ml, 3: LIF 25 ng/ml, 4: IL-6 10 ng/ml, 5: bFGF 25 ng/ml, 6: IL-1β 5 ng/ml.

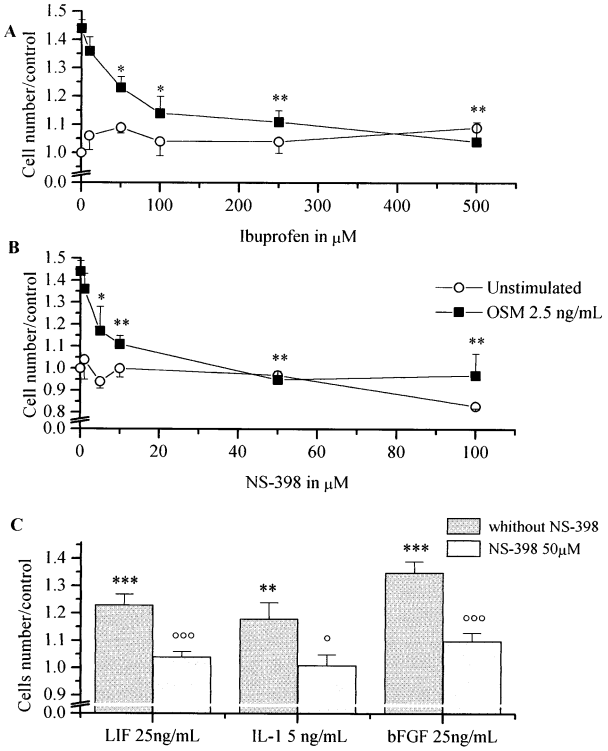

Fig. 3. Effects of COX-2 inhibitors on OSM-, LIF-, IL-1β- and bFGF-induced HMEC-1 proliferation. (A) and (B) 50×10^3 HMEC-1 cells/well were seeded in a 24 well plate and incubated with increasing concentrations of ibuprofen (A) or NS-398 (B) in the presence (closed square) or in the absence (open circle) of 2.5 ng/ml OSM. At day 5, cells were detached by trypsin and then counted with a hemocytometer. Results of five experiments in duplicate are expressed as the ratio of the number of cells counted over the number of cells counted in the control ± S.E.M. ($^*P<0.05$, $^{**}P<0.005$ as compared with OSM-treated cells, $n=5$). (C) Cells were seeded as in (A) and incubated with 25 ng/ml LIF, 5 ng/ml IL-1β or 25 ng/ml bFGF in the absence or the presence of 50 μM NS-398 and then counted after a 5 day incubation. Results of five experiments in duplicate are expressed as the ratio of the number of cells counted over the number of cells counted in the control ± S.E.M. ($^{**}P<0.005$, $^{***}P<0.001$ as compared with control cells, $^{\circ}P<0.05$, $^{\circ\circ\circ}P<0.001$ as compared with NS-398-untreated cells, $n=5$).

Co-incubation of 2.5 ng/ml OSM with ibuprofen (1–500 μM) for 5 days showed a dose-dependent decrease in OSM-induced proliferation, reaching a plateau at 300 μM ibuprofen (Fig. 3A). In contrast, in the absence of OSM, HMEC-1 proliferation was not affected by ibuprofen.

The effect was more pronounced with NS-398 (0.1–100 μM), as we observed a dose-dependent inhibition of OSM-induced proliferation reaching a plateau at 50 μM (Fig. 3B).

We also evaluated whether NS-398 (50 μM) could modify the increase in cell proliferation induced by LIF, IL-1β or bFGF. Fig. 3C shows that COX-2 inhibition totally prevented IL-1β- or LIF-induced HMEC-1 proliferation and inhibited up to 70% of bFGF-induced proliferation.

3.5. Effect of NS-398 on OSM-induced HMEC-1 migration

As OSM is a chemoattractant for HMEC-1, we analyzed the effect of NS-398 on OSM-induced migration. The chemo-

attractant property of OSM was tested in the Transwell system with an 8 μm pore diameter membrane. A 24 h incubation of HMEC-1 with 2.5 ng/ml OSM induced a significant increase in migration as compared with control, which was partially inhibited by 50 μM NS-398 (2.49 ± 0.03-fold the control for 2.5 ng/ml OSM alone versus 1.51 ± 0.12-fold for OSM plus NS-398, $P < 0.01$, $n = 4$). The chemoattractant effect of 25 ng/ml LIF was completely inhibited by 50 μM NS-398 (1.67 ± 0.04-fold the control for 25 ng/ml LIF alone versus 1.15 ± 0.02-fold for LIF plus NS-398, $P < 0.01$, $n = 4$).

3.6. *Effect of COX inhibitor on OSM-induced angiogenesis in the rabbit cornea*

OSM at 100 ng/implant promoted new vessel growth processing from the limbus to the implant (score = 3 ± 0, $n = 4$ Fig. 4A), while a treatment with 0.03% flurbiprofen three times a day for 7 days inhibited almost completely OSM-induced angiogenesis (score = 1 ± 0.5, $n = 4$, Fig. 4B) ($P < 0.005$).

4. Discussion

In a previous study, we analyzed the proliferative effect of OSM and related cytokines (LIF and IL-6) on ECs from different origins and species [3]. OSM induced a strong proliferative effect on microvascular ECs (HMEC-1), which was stronger than that induced by the two angiogenic factors used as reference, bFGF and VEGF. Furthermore, we showed that OSM, LIF and IL-6, when added to the lower compartment of a modified Boyden chamber (Transwell), induced a chemotactic effect, as shown by increased cell migration through a polycarbonate membrane. We therefore proposed that this angiogenic effect of OSM might be involved in the inflammatory process and in progression of atherosclerosis. This was supported by the presence of activated monocytes/macrophages in an advanced lesion of atherosclerosis. The aim of the present work was to identify possible molecular effector(s) of this OSM effect on angiogenesis which could be involved in atherosclerotic plaque fragilization.

Among the mechanisms involved in OSM-induced EC proliferation, we further observed that OSM stimulates slightly the secretion of VEGF on HMEC-1, VEGF is known to induce proliferation of ECs. However, as a neutralizing VEGF antibody only inhibited partially its proliferative effect, we assumed that the proliferative effect of OSM is not totally due to the secretion of VEGF [3].

In this work, we demonstrate that OSM markedly induces COX-2 expression in HMEC-1. COX-2 mRNA expression in HMEC-1 was increased after 3 h of incubation with OSM and remained increased after 24 h, leading to a sustained expression of COX-2 in HMEC-1. The increase in COX-2 mRNA was accompanied by a concomitant expression of COX-2 protein after 24 h to the same extent as induced by IL-1β.

In a previous study, we have shown that the angiogenic effect of OSM and LIF was specific for microvascular ECs and was not observed on macrovascular ECs, HUVECs. The fact that neither OSM nor LIF induced COX-2 mRNA in HUVECs suggested that COX-2 might mediate the angiogenic activity of OSM.

Inhibitors of COX-2 (ibuprofen, NS-398) totally inhibit OSM and LIF proliferative effects on microvascular ECs, supporting our hypothesis that COX-2 is a critical regulatory molecule for OSM- and LIF-induced angiogenesis.

Therefore, OSM and LIF induced angiogenesis activity by a mechanism similar to that of bFGF, the in vivo and in vitro activity of which have been demonstrated to be COX-2-dependent [6,12]. On HMEC-1 cells, we also showed an inhibitory effect of NS-398 on bFGF-induced proliferation, as observed with LIF and OSM. Thus, microvascular ECs produce COX-2 in response to OSM, LIF or bFGF, which appears to be responsible for angiogenic activity of these molecules. In a rabbit corneal pocket model, we also demonstrated the role of COX in OSM-induced angiogenesis as the formation of neovessels was totally inhibited by local administration or flurbiprofen. Our findings showing the role of COX-2 in angiogenesis are different from those of Tsujii et al. [5]. These authors employed an EC/colon carcinoma cell co-culture model to explore the role of COX-2 in angiogenesis. They showed that COX-2 activity in colon carcinoma promotes angiogenesis by production of angiogenic factors, including bFGF and VEGF. Nevertheless, they did not find any COX-2 production in the ECs. In this model, they used HUVECs, which in our hands produce COX-2 in response to bFGF. However, as these authors used a co-culture model, other factors secreted by cancer cells could explain this discrepancy. They observed an increase in TGF-β1, which is known to decrease EC proliferation in vitro [13].

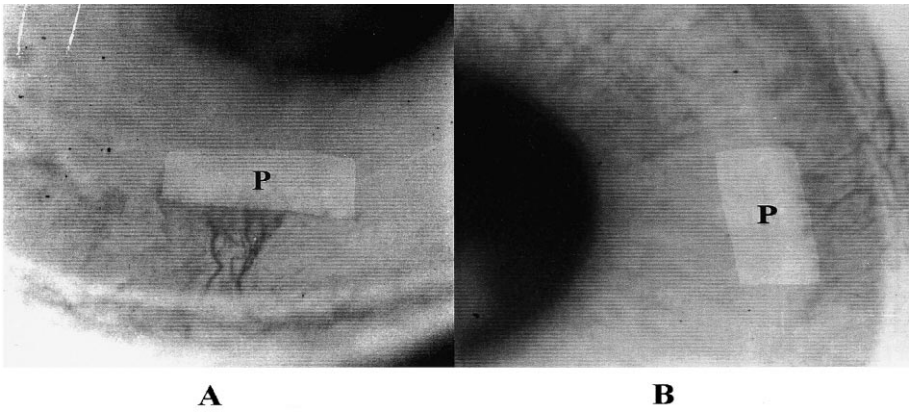

Fig. 4. Photographs of rabbit cornea with an implant containing OSM at 100 ng/implant (A) or OSM at 100 ng/implant+flurbiprofen treatment (B), 7 days after implantation (P: implant).

IL-1β, which is one of the most potent inducers of COX-2 expression, induced only a poor proliferative effect on HMEC-1, as shown in Fig. 3. This suggest that COX-2 is required for but is not sufficient to promote proliferation.

This is not yet explained, but it is already shown that COX-2 products need to cooperate with other mediators such as iNOS in the inflammatory response [14].

In conclusion, PG products of COX-2 are important angiogenic factors downstream of other angiogenic factors, because both OSM and LIF, as well as bFGF, could up-regulate COX-2 expression and, in consequence, induce angiogenesis by promoting EC proliferation and migration.

Our results underline the fact that the inhibition of COX-2 activity could be a major therapeutic strategy to limit angiogenesis and, by consequence, the diseases in which angiogenesis is a key process, such as atherosclerosis, chronic inflammation and cancer.

Acknowledgements: This work was supported by grants from the Fondation de France and from the Groupement des Entreprises Françaises dans la Lutte contre le Cancer (GEFLUC). J. Pourtau is a recipient of a fellowship from the Ministère de l'Education Nationale, de la Recherche et de la Technologie of France. The authors thanks Mme Legrand for her help in the realization of this work ands Mr. Meideros for his editorial assistance.

References

[1] Vasse, M., Collet, J.-P., Soria, J., Mirshahi, S.-S., Vannier, J.-P. and Soria, C. (1994) Thromb. Res. 75, 349–352.
[2] Modur, V., Feldhaus, M.J., Weyrich, A.S., Jicha, D.L., Prescott, S.M., Zimmerman, G.A. and McIntyre, T.M. (1997) J. Clin. Invest. 100, 158–168.
[3] Vasse, M., Pourtau, J., Trochon, V., Muraine, M., Vannier, J.-P., Lu, H., Soria, J. and Soria, C. (1999) Arterioscler. Thromb. Vasc. Biol. (in press).
[4] Smith, W.L., Garavito, R.M. and DeWitt, D.L. (1996) J. Biol. Chem. 271, 33157–33160.
[5] Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M. and DuBois, R.N. (1998) Cell 93, 705–716.
[6] Majima, M., Isono, M., Ikeda, Y., Hayashi, I., Hatanaka, K., Harada, Y., Katsumata, O., Yamashina, S., Katori, M. and Yamamoto, S. (1997) Jpn. J. Pharmacol. 75, 105–114.
[7] Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T. and Hla, T. (1994) J. Biol. Chem. 269, 11769–11775.
[8] Ades, E.W., Candal, F.J., Swerlick, R.A., George, V.G., Summers, S., Bosse, D.C. and Lawley, T.J. (1992) J. Invest. Dermatol. 99, 683–690.
[9] Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, R. (1997) J. Clin. Invest. 352, 2745–2756.
[10] Bradford, M. (1976) Anal. Biochem. 72, 248–250.
[11] Bishop-Bailey, D., Pepper, J.R., Larkin, S.W. and Mitchell, J.A. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1655–1661.
[12] Kage, K., Fujita, N., Oh-hara, T., Ogata, E., Fujita, T. and Tsuruo, T. (1999) Biochem. Biophys. Res. Commun. 254, 259–263.
[13] Sutton, A.B., Canfield, A.E., Schor, S.L., Grant, M.E. and Schor, A.M. (1991) J. Cell. Sci. 99, 777–787.
[14] Baker, C.S.R., Hall, R.J.C., Evans, T.J., Pomerance, A., Maclouf, J., Creminon, C., Yacoub, M.H. and Polak, J.M. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 646–655.