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Effect of aescine on hypoxia-induced neutrophil adherence to umbilical vein endothelium

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Abstract

Although venous stasis due to blood stagnation in lower limbs has been recognised as an important etiological factor for the development of varicose veins, the mechanism linking this ischemic situation to the modifications of the venous wall in varicose veins is still unclear. There is evidence that the activation of the endothelium during blood stasis and its subsequent cascade of interactions with other cell types could alter the structure of the vein wall and could possibly be at the origin of the disease. While phlebotonic drugs are often used to improve symptoms in chronic venous insufficiency, their precise mechanism of action is not well understood. We now tested aescine (Reparil i.v. form) in an ex vivo model which mimics this situation, i.e., perfused human umbilical vein exposed to hypoxic conditions. To study the effect of aescine on neutrophil activation and adhesion to the endothelium, human umbilical veins were incubated under hypoxic conditions with or without aescine and the interactions between the endothelium and neutrophil-like cells, HL60, were investigated. We observed that a large number of HL60 became adherent to the endothelium of veins after 2 h hypoxia and that these adherent HL60 were activated: they released high amounts of superoxide anion and of leukotriene B_4. Aescine (250 ng/ml or 0.22 μM) was shown to markedly inhibit HL60 adherence to hypoxic endothelium. By decreasing the number of adherent HL60, aescine also decreased the subsequent production of superoxide anion and of leukotriene B_4. Scanning electron microscopy confirmed the increased HL60 adherence to the endothelium, as well as the inhibitory effect of aescine. These results support results of in vitro studies on isolated endothelial cells in which aescine was shown to inhibit the hypoxia-induced activation of endothelial cells and the subsequent increased adherence of neutrophils. In vivo, the activated and infiltrated leukocytes release free radicals, chemotactic molecules such as leukotriene B_4 and proteases which then can degrade the extracellular matrix. These processes could contribute to alterations of the venous wall similar to those observed in varicose veins. By maintaining an intact endothelium during in vivo blood stasis in the lower limbs and preventing neutrophil recruitment, adherence and activation, aescine could prevent the resulting alterations of the venous wall. These results could explain at least in part the potential benefit of the drug in the prevention of venous insufficiency. © 1998 Elsevier Science B.V.

Keywords: Hypoxia; Endothelium; Neutrophil adherence; Neutrophil activation; Aescine; Reparil

1. Introduction

The etiology of chronic venous insufficiency is not yet completely understood. Both constitutive (such as genetic factor, age, sex, etc.) and environmental factors have been shown to be involved in the development of this disease. The appearance of varicose veins seems to be linked to a reduction of blood flow which is frequently observed in the lower limbs of patients (Browse and Brunand, 1982). On the basis of this observation, a new hypothesis has been proposed for the development of varicose veins. During blood stasis, ischemic conditions develop in which hypoxia is an important parameter, itself able to lead to a strong activation of the endothelial cells (Arnould et al., 1992; Michiels et al., 1993). Such an activated endothelium releases growth factors for smooth muscle cells (Michiels et al., 1994b) and synthesises inflammatory mediators leading to the recruitment, adherence and activation of neutrophils (Arnould et al., 1993, 1994). Both effects could lead to alterations in the venous wall similar to the ones observed in varicose veins such as smooth muscle cell proliferation and collagen modifications (Michiels et al., 1994a, 1996).

Several therapeutic approaches are now available for the treatment of this disease and among them, phlebotonic

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drugs are useful to prevent the worsening of the disease. Aescine (a triterpenic glucoside: $C_{55}H_{87}O_{24}$) is frequently used in the treatment of chronic venous insufficiency. Several of its pharmacological properties tested on animal models are well-known. This drug was shown to have anti-inflammatory activity (Rothkopf-Ischebeek and Vogel, 1980), to prevent edema formation by reducing microvascular permeability (Vogel et al., 1970) and to exert venotonic activity by increasing venous tone (Annoni et al., 1979). All these effects may partly explain the beneficial action of aescine in patients with venous insufficiency. However, its precise mechanism of action remains poorly understood.

An in vitro model in which endothelial cells were exposed to hypoxic conditions was used to show that aescine inhibits, in a dose-dependent manner, the hypoxia-induced activation of endothelial cells as evidenced by the protection against the hypoxia-induced decrease of ATP content, the increase in phospholipase A_2 activity and the increase in neutrophil adherence (Arnould et al., 1995b). In order to extrapolate the effects of aescine obtained with endothelial cells in culture to those one could expect in vivo during aescine therapy, it is very important to verify that these effects are relevant to this in vivo situation. While endothelial cells in culture are very useful, the phenotype of the cultured endothelial cells is known to be slightly different from the one observed in vivo. There are two main reasons for this change in phenotype: first, endothelial cells in culture are stimulated by foetal serum and are proliferating, contrary to the in vivo quiescent state, and secondly, since the cells are no longer undergoing shear stress, the expression of several genes regulated by this stress is changed in culture (Gimbrone, 1997). It was thus very important to verify that the inhibition by aescine of the hypoxia-induced activation of endothelial cells as well as of the subsequent increase in neutrophil adherence could also be obtained with a fresh, complete and well-differentiated endothelium. Using umbilical veins, the results now described show that this is indeed the case. In order to demonstrate the activity of aescine in this model, several biochemical parameters were followed: the adherence of neutrophil assessed by the number of chromium-51-labeled neutrophil-like cells associated to the umbilical vein, the production of superoxide anions and the synthesis of leukotriene B_4 by neutrophil-like cells adherent to the venous endothelium. Furthermore, the morphology was studied, using scanning electron microscopy. All experiments were carried out with 250 ng/ml of aescine since this concentration was previously reported to be optimal in the in vitro model of hypoxia with endothelial cells in culture.

2. Materials and methods

Aescine is the active molecule of Reparil from Madaus Pharma (Köln, Germany). Aescine is a triterpenic glucoside ($C_{55}H_{87}O_{24}$) present as the sodium salt form in the Reparil (i.v. form) used in this study. The drug was solubilised directly in the incubation buffer, with 250 ng/ml corresponding to 0.22 μM.

2.1. Perfusion of the umbilical veins

All the manipulations were carried out at 37°C. Human umbilical cords freshly harvested (less than 24 h after birth) and stored in physiological solution (4 mM KCl, 140 mM NaCl, 10 mM HEPES, 1 mM glucose, 100 μg/ml streptomycin, 100 μg/ml penicillin, pH 7.35) were rinsed with sterile PBS (phosphate-buffered saline), cut into three equal pieces: one to be perfused at 1 ml/min with a peristaltic pump (Peristaltic pump P1, Pharmacia, Uppsala, Sweden) for 5 min with normoxic HBSS (Hanks balanced salt solution: 140 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 4.3 mM $NaH_2PO_4 \cdot 2H_2O$, 5.5 mM D-glucose, 1 mM $CaCl_2$, pH 7.4, $PO_2 = 130$ mmHg), another for 5 min with hypoxic HBSS (50 ml of HBSS gassed for 60 min with 100% N_2, $PO_2 = 10$ mmHg) and a third perfused for 5 min with hypoxic HBSS containing aescine at 250 ng/ml (10 ml of HBSS gassed for 10 min with 100% N_2, $PO_2 = 10$ mmHg). The end of the vessel was then clamped, the vein was filled with 5 ml of normoxic or hypoxic HBSS and the perfusion was stopped for 120 min.

2.2. HL60 labeling and adherence assay

The HL60 cell line (Peripheral Blood Human-Promyelocyte Leukemia) is a neutrophil cell line. HL60 are phagocytotic cells which respond to chemotaxis and express receptors for Fc fragment and complement (Collins et al., 1977). They are cultivated in RPMI + 10% fetal calf serum. For labeling, cells at the density of 10×10^6 cells/ml were incubated with 20 μCi ^{51}Cr/ml (Specific activity = 250–500 mCi/mg chromium, Amersham Laboratories, Amersham, UK) in HBSS with calcium and magnesium for 60 min at room temperature, washed 3 times and then suspended at 5×10^6 cells/ml in HBSS.

For the adherence assay, the clamps closing the vein were removed in order to eliminate the incubation solution and the vein was then perfused with a suspension of ^{51}Cr-labeled HL60 at 5×10^6 cells/ml for 5 min at 1 ml/min, clamped and filled with 1 ml of the HL60 suspension. Afterwards, the vein was again perfused at 1 ml/min with HBSS alone for 5 min to remove non-adherent cells. The cord was then collected, cut into small pieces, homogenised for 2 min in 2×10 ml of HBSS with an Ultraturax (OmniMixer, DuPont Instrument, Newton, CT) and the radioactivity was counted in a gamma counter. The results are expressed as neutrophil-like cells number per centimeter of cord.

2.3. Isolation and labeling of human neutrophils

Human neutrophils were purified from blood of healthy donors by the procedure of Boyum (1976). In brief, 30 ml of venous anticoagulated blood from normal subjects was mixed with 5 ml of 6% dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) and allowed to sediment at 20°C for 60 min. After hypotonic lysis of erythrocytes performed with NaCl 0.2% for 1 min, the cells were centrifuged 20 min at 1000 rpm on Lymphoprep (Nycomed Pharma, Oslo, Norway). Labeling procedure and adherence assay were performed as described for HL60.

2.4. Estimation of superoxide anion production

The generation of superoxide anion from adherent neutrophil-like cells was assessed by the superoxide dismutase inhibitable reduction of ferricytochrome *c*. Neutrophil-like cells (5×10^6 cells/ml) resuspended in ferricytochrome *c* solution (1 mg/ml in HBSS) were co-incubated with normoxic or hypoxic vein preincubated or not with aescine at 250 ng/ml for 5 min. After this incubation, the neutrophil-like cells suspension was recovered, placed at 4°C and centrifuged 5 min to eliminate the cells. Absorbance of the cytochrome *c* solution was determined spectrophotometrically at 550 nm and compared to results of similar tests containing superoxide dismutase (300 U/ml). The data were converted into nanomoles of $O_2^{\cdot(-)}$ ml^{-1} min^{-1}.

2.5. Leukotriene B_4 assay

Leukotriene B_4 was measured using an Enzyme ImmunoAssay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). We performed the assay as described in the kit. Briefly, this assay is based on the competition between free leukotriene B_4 and a leukotriene B_4 tracer (leukotriene B_4 linked to an acetylcholinesterase molecule). The tracer is quantitated by measuring its acetylcholinesterase activity with Ellman's reagent consisting of acetylcholine and 5,5′-dithio-bis(2-nitrobenzoic acid). Hydrolysis of acetylcholine produces thiocholine which reacts with 5,5′-dithio-bis(2-nitrobenzoic acid), which has strong absorbance at 412 nm. Absorbance was then converted into picogram per milliliter using the standard calibration curve.

2.6. Scanning electron microscopy

For scanning electron microscopy, after the last wash, which removes non-adherent neutrophils, the umbilical vein and the adherent neutrophils were fixed by perfusing the vein with cacodylate buffer (0.1 M, pH 7.4) containing 1.5% glutaraldehyde for 20 min at 4°C, then the cord was cut into pieces 1 cm in length. These pieces were then placed in the cacodylate–glutaraldehyde buffer overnight. They were dehydrated for 15 min in 30, 50, 70 and 90% acetone at 4°C and then in 100% acetone at room temperature. They were finally prepared by critical-point drying, mounted on an aluminium stub and covered with a thin layer of gold (20 nm), then examined in a scanning electron microscope (Philips, XL-20, Eindhoven, The Netherlands).

2.7. Statistical analysis

The data are presented as means $\pm$ 1 S.D. When different cords were used in different experiments, the means were compared using a one-way analysis of variance and Scheffé's contrasts.

3. Results

3.1. Effect of aescine on HL60 adherence to human umbilical vein endothelium

The aim of the study was to investigate the effect of aescine on the interactions of neutrophils with human umbilical veins after incubation under hypoxic conditions. For this purpose, umbilical veins were incubated 2 h under hypoxic conditions with or without aescine at 250 ng/ml with a matched control maintained under normoxic conditions. The veins were then perfused with a suspension of HL60 which were incubated in the vein for 5 min. After washing the vein to remove non-adherent cells, adherence of HL60 to the endothelium was assayed as well as their activation. Morphological scanning electron microscopy was performed in parallel.

A neutrophil-like cell line, HL60, was used throughout the work. These cells are phagocytic cells with properties similar to those of neutrophils (Collins et al., 1977). They are often used in studies investigating adherence of these leucocytic cells to endothelium under various conditions (Bevilacqua et al., 1985). The effect of aescine on neutrophil-like cell adherence to the umbilical vein endothelium was first studied. Fig. 1 shows that the number of neutrophil-like cells associated with the vein increased markedly under hypoxic conditions as compared to normoxic conditions, reaching 200 000 to 250 000 neutrophil-like cells/cm of cord. It must be noted that baseline adherence of neutrophil-like cells to normoxic endothelium was lower but much more variable from one cord to the other. This variability between different umbilical cords has been reported frequently (DeCaterina et al., 1985; McIntyre et al., 1985; Baenziger et al., 1979).

However, in each experiment, aescine strongly inhibited the adherence of neutrophil-like cells to the umbilical vein endothelium incubated under hypoxic conditions. Inhibition of 152, 181, 97 and 173% was obtained in four independent experiments. Percentage inhibition was calculated as the decrease in the number of adherent neutrophils under hypoxic conditions due to the presence of aescine compared to the number under normoxic conditions. An

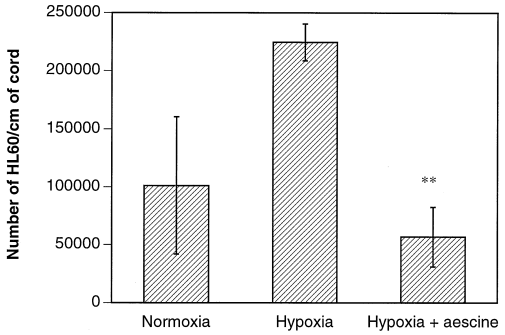

Fig. 1. Effect of aescine on HL60 adherence to human umbilical vein endothelium. Matched veins were exposed 120 min to hypoxia with or without aescine at 250 ng/ml or to normoxia, then perfused with a suspension of ^{51}Cr-labeled unstimulated HL60, co-incubated with these cells for 5 min and finally washed 5 min to remove non adherent HL60. Results are expressed as HL60 number per centimeter of the veins, as means ± 1 S.D. from four independent experiments. $**$ Significantly different from hypoxia with $P < 0.01$ using a one-way analysis of variance with Scheffé's contrasts.

inhibition percentage higher than 100% means that aescine inhibited the hypoxia-induced adherence to a value lower than the baseline adherence for normoxic conditions. When the means of the four experiments were compared, the effect of aescine was statistically significant ($P < 0.01$). On the other hand, aescine did not inhibit the adherence of neutrophils to normoxic veins: $170\,514 \pm 37\,445$ neutrophils/cm of cord ($n = 3$) was obtained in normoxic veins while $196\,066 \pm 10\,787$ neutrophils/cm of cord was found in normoxic veins incubated in the presence of 250 ng/ml of aescine. These results indicate that aescine only inhibited the hypoxia-induced neutrophil adherence to the endothelium and have no nonspecific effects on normoxic endothelium.

Aescine is thus able to completely inhibit the effect of hypoxia on HL60 adherence to the umbilical vein endothelium. HL60 and neutrophil have similar properties, we then wanted to verify the effect of aescine with purified human neutrophils. Fig. 2 shows that hypoxia was able to strongly increase neutrophil adherence to umbilical vein endothelium as was observed with HL60. The percentage increase in adherence observed with neutrophils corre-

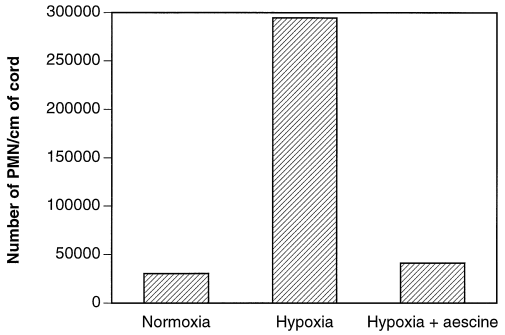

Fig. 2. Effect of aescine on human neutrophil adherence to human umbilical vein endothelium. Matched veins were exposed 120 min to hypoxia with or without aescine at 250 ng/ml or to normoxia, then perfused with a suspension of ^{51}Cr-labeled unstimulated neutrophils, co-incubated with these cells for 5 min and finally washed 5 min to remove non-adherent neutrophils. Results are expressed as neutrophil (PMN) number per centimeter of the veins, as means from two independent experiments.

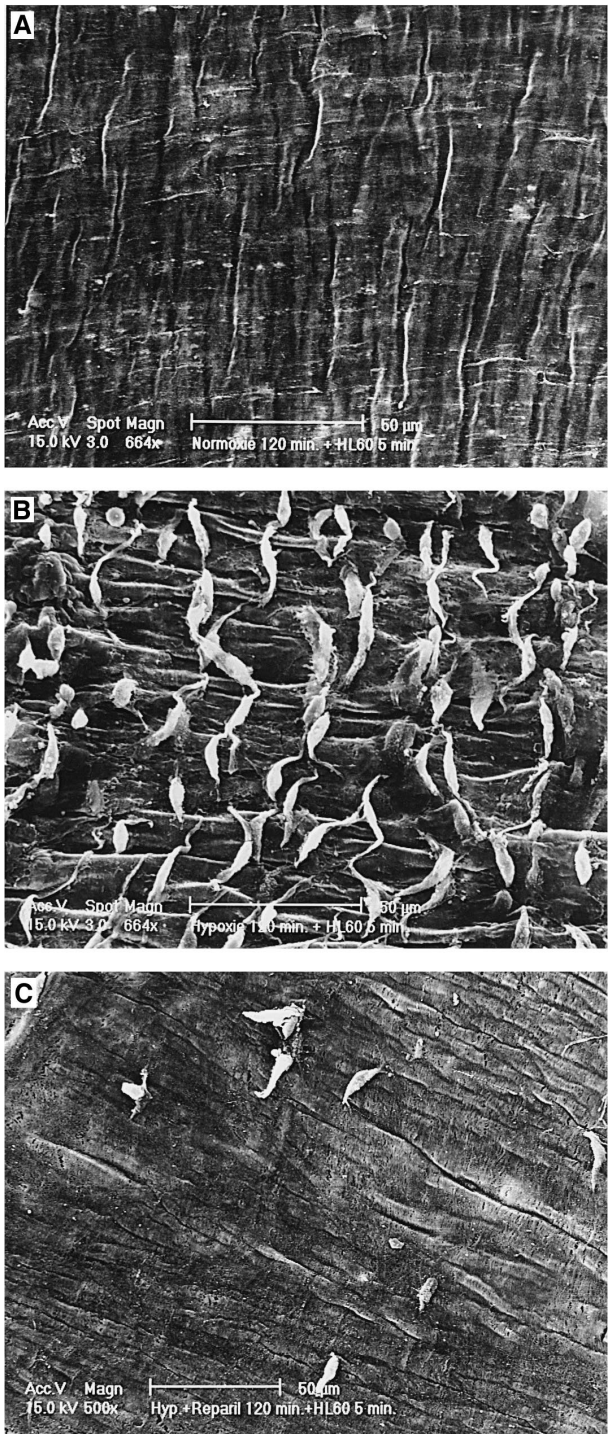

Fig. 3. Micrographs of HL60 adherent to human umbilical vein endothelium, scanning electron microscopy. Matched veins were exposed 120 min to hypoxia with or without aescine (C or B) or to normoxia (A), then perfused with a suspension of HL60, co-incubated with these cells for 5 min and finally washed 5 min to remove non-adherent HL60. The veins were cut transversally after fixation and observed by scanning electron microscopy (original magnification = 500×).

sponds to that with HL-60. As with HL60, aescine completely inhibited (96%) this hypoxia-induced adherence.

The increase in HL60 adherence to the endothelium of hypoxic umbilical vein was confirmed by scanning electron microscopy. Very few HL60 were seen to adhere to the umbilical endothelium when the vein was kept under normoxic conditions (Fig. 3A). On the other hand, numerous adherent HL60 were observed on the endothelium of veins incubated under hypoxic conditions (Fig. 3B). In addition, these HL60 showed morphological alterations, such as a ruffled surface or polarised shape, consistent with an activated state. The micrographs shown in Fig. 3C also clearly demonstrate that aescine (250 ng/ml) strongly inhibited HL60 adherence to the umbilical vein endothelium following hypoxic incubation and prevented their activation. No loss and no sign of mortality of the endothelial cells was evidenced by scanning electron microscopy when the lumen of the vein was observed after perfusion under either normoxic or hypoxic conditions. We thus concluded that hypoxia greatly increased HL60 adherence to the umbilical vein endothelium which was totally inhibited by aescine.

3.2. Effect of aescine on activation of HL60 adherent to the human umbilical vein endothelium

Previous results obtained in vitro and the micrographs presented in Fig. 3B showed that HL60 become activated when adherent to hypoxic endothelial cells. The activated state of adherent HL60 was investigated in more details when the cells were incubated with hypoxic umbilical vein endothelium: the production of superoxide anion by NADPH oxidase and the synthesis of leukotriene B_4 resulting from the activation of the 5-lipoxygenase were assayed. Fig. 4 shows that superoxide anion production was greater for HL60 adherent to the endothelium of veins incubated under hypoxia than for HL60 adherent to normoxic veins. Superoxide anion production by HL60 co-incubated in the vein was 2-fold higher (mean from three independent experiments) after hypoxic incubation than after normoxic incubation. This greater superoxide production was actually due to HL60 activation and not to reoxygenated endothelial cells since the cytochrome *c* reduction in the solution perfused without HL60 in a vein incubated under hypoxia was not greater than that in the control normoxic vein with HL60 (compare 4th column to 1st column in Fig. 4). The incubation with HL60 was limited to 5 min in order to avoid such reoxygenation. In each experiment, aescine strongly prevented the activation of HL60 co-incubated with the umbilical vein endothelium previously incubated under hypoxic conditions. Inhibition of 86, 63 and 131% was obtained in three independent experiments. When the means of the three experiments were compared, the effect of aescine was statistically significant, with $P < 0.05$.

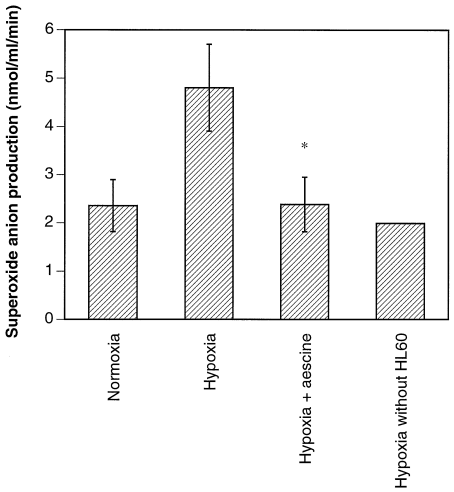

Fig. 4. Quantification of the activation of HL60 adherent to human umbilical vein endothelium. Estimation of the superoxide anion production by HL60 (5×10^6 cells) co-incubated for 5 min in umbilical veins previously incubated 120 min under hypoxia with or without aescine at 250 ng/ml or maintained in normoxia. Superoxide anion production was measured by ferricytochrome *c* reduction. Results are expressed as nmol ml^{-1} min^{-1} as means $\pm$ 1 S.D. from three independent experiments. * Significantly different from hypoxia with $P < 0.05$ using a one-way analysis of variance with Scheffé's contrasts.

Some controls were performed using purified neutrophils. Production of superoxide anion by neutrophils in normoxic or hypoxic veins was compared to the production in normoxic or hypoxic veins alone: 1.420 and 4.114 nmol min^{-1} ml^{-1} was obtained in the presence of neutrophils in comparison to 1.133 and 0.752 nmol min^{-1} ml^{-1} in the absence of neutrophils for respectively normoxic and hypoxic conditions. These results indicate that neither hypoxic or normoxic endothelial cells nor neutrophils incubated in normoxic vein produced significant amount of superoxide anion. Only neutrophils incubated in hypoxic veins produced a marked quantity of superoxide anion. The direct effect of aescine on neutrophil activation by a soluble stimulus was also studied by incubating neutrophils with 10^{-7} M fMLP (formyl–methionyl–leucinyl–phenylalanine) for 5 min in the absence or in the presence of 250 ng/ml of aescine. The production of superoxide anion was 0.870 ± 0.230 nmol min^{-1} ml^{-1} ($n = 3$) for unstimulated neutrophils, 3.384 ± 0.133 for neutrophils stimulated with fMLP and 3.571 ± 0.044 for neutrophils stimulated with fMLP in the presence of aescine. These results indicate that aescine does not inhibit neutrophil or HL60 activation through a soluble stimulus but rather by reducing the adherence process.

Another metabolic pathway which is activated when HL60 are stimulated is the activation of phospholipase A_2 and of 5-lipoxygenase, leading to the synthesis of leukotriene B_4. The leukotriene B_4 production by HL60 was enhanced when these cells were co-incubated with the umbilical vein after a 120 min hypoxia incubation (Fig. 5). Leukotriene B_4 concentration in conditioned media after hypoxia (479.9 pg/ml) was 6.3-fold higher than the con-

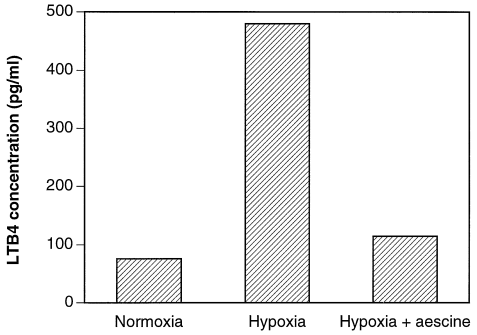

Fig. 5. Quantification of the activation of HL60 adherent to human umbilical vein endothelium. Estimation of leukotriene B_4 synthesis by HL60 (5×10^6 cells) co-incubated for 5 min in umbilical veins previously incubated 120 min under hypoxia with or without aescine at 250 ng/ml or maintained in normoxia. Leukotriene B_4 production was measured in an EIA. Results are expressed as picogram per milliliter.

centration for control conditions (76.2 pg/ml). When the vein was incubated under hypoxic conditions with aescine at 250 ng/ml, the concentration measured after HL60 co-incubation was only 114.4 pg/ml which corresponds to an inhibition of 90%. This percentage is very similar to the inhibition percentage obtained for superoxide anion production.

The values for both parameters, as well as the morphology of these adherent HL60, thus indicate that HL60 which adhere to the endothelium of umbilical veins incubated in hypoxic conditions are more strongly activated than those adhering to normoxic veins. Furthermore, aescine strongly inhibited HL60 adherence and thus prevented their subsequent activation when they were co-incubated with the endothelium of umbilical veins previously exposed to hypoxia.

4. Discussion

In venous stasis, blood stagnation and the resulting hypoxia have long been recognized to be involved in the development of varicose veins (McEwan and McArdle, 1971). However, the mechanism linking this hypoxia to the development of the modifications observed in the venous wall and the potential mechanism of action of phlebotonic drugs remain poorly understood.

The unique position of the endothelium interposed between the intravascular space and tissues allows endothelial cells to play a key role in regulating vascular hemostasis. The active processes of endothelial cells include secretion (Jaffe, 1985), modulation of coagulation (Stern et al., 1991) and interactions with smooth muscle cells (Lusher, 1993) and inflammatory cells (Pober and Cotran, 1990). Any disturbance of endothelial cell metabolism induced, for example, by an ischemic or hypoxic event would thus have severe consequences for vascular hemostasis and more particularly for interactions of endothelial cells with leukocytes. One hypothesis for the etiology of chronic venous insufficiency proposed that trapped activated leukocytes in the venous wall could eventually lead to tissue alterations. Michiels et al. (1994a, 1996) recently proposed that at least one initial event in varicose vein development could be activation of the endothelium by hypoxic conditions occurring during blood stasis. Indeed, hypoxia-activated endothelial cells release growth factors for smooth muscle cells (Michiels et al., 1994a) and inflammatory mediators which induce the first steps of neutrophil infiltration, such as their recruitment, adherence and subsequent activation (Arnould et al., 1993, 1994).

Aescine is widely used in the treatment of chronic venous insufficiency and several clinical trials have shown that aescine therapy had significant superior beneficial effects versus placebo in the treatment of symptoms related to this disease (Diehm et al., 1996). Its effects was known on capillary filtration, microvascular permeability and edema formation suggesting that endothelial cells could be a target for this molecule, but its precise mechanism of action is still unclear.

In this work, we studied the influence of hypoxia on the interactions between HL60 and the endothelium using perfused human umbilical vein. Previous results showed that neutrophils adhere strongly to the endothelium of hypoxic veins compared to that of normoxic veins (Arnould et al., 1995a). We now showed that HL60 also increased their adherence to the endothelium of umbilical veins after hypoxic incubation. These adherent HL60 were activated and released large amounts of superoxide anions and leukotriene B_4. Previous works with isolated human umbilical vein endothelial cells had demonstrated that hypoxia strongly activates human umbilical vein endothelial cells (Arnould et al., 1992) which then release chemotactic factors for neutrophils (unpublished results) as well as prostaglandins (Michiels et al., 1993) and synthesize PAF (platelet-activating factor). These hypoxic human umbilical vein endothelial cells also become much more adhesive for neutrophils, this process being PAF-dependent and mediated by CD18/CD11b–ICAM-1 (intercellular adhesion molecule-1) interactions (Arnould et al., 1993). The same intercellular interactions have been shown to be involved in the hypoxia-induced neutrophil adherence to umbilical veins (Arnould et al., 1995a). The adherence of neutrophils to hypoxic human endothelial cells leads to their activation, with increased synthesis of superoxide anion and leukotriene B_4 (Arnould et al., 1994).

The present results clearly demonstrated that aescine, 250 ng/ml, which corresponds to 0.22 μM, was able to strongly inhibit the adherence of HL60 to the human umbilical vein endothelium after a 120 min incubation of the vein under hypoxic conditions. By inhibiting the HL60 adherence process, aescine also prevented the subsequent activation of these adherent cells. A decrease of about 90% was obtained for both parameters tested, i.e., superoxide anion production and the synthesis of leukotriene B_4. All

these results were confirmed by morphological observations by scanning electron microscopy. It was previously shown that aescine prevented in a dose-dependent manner the hypoxia-induced activation of human endothelial cells evidenced by the inhibition of hypoxia-increased phospholipase A_2 activity as well as neutrophil adherence, probably by maintaining the ATP content of human umbilical vein endothelial cells during hypoxia (Arnould et al., 1995b). It is thus probable that, by preventing the hypoxia-induced activation of the endothelial cells of the umbilical vein as it does in vitro, aescine can prevent the hypoxia-induced adherence of HL60 to the endothelium of the veins under these conditions.

Such effects of aescine in vivo could be very important since they could explain the inhibition of the neutrophil infiltration in the venous wall. This would then block the subsequent inflammation as well as the edema formation. Aescine would also prevent neutrophil plugging in the microcirculation because the protected endothelium remains non-adhesive for neutrophils, thus facilitating their circulation in the blood vessel. This drug has indeed been found to be clinically useful to facilitate microcirculatory flow, which may be beneficial for improving symptoms. These data suggest that protection of the endothelium by aescine during blood stasis, which in turn prevents the adherence, activation and infiltration of neutrophils and the subsequent damage to the venous wall is at least in part responsible for the beneficial action of this drug in the treatment of venous insufficiency.

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