Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]
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GM-CSF: A STRONG ARTERIOGENIC FACTOR
ACTING BY AMPLIFICATION OF MONOCYTE FUNCTION

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CHAPTER 6

Abstract

Background: We investigated the role of the colony stimulating factor for monocytes, GM-CSF, to test the hypothesis whether prolongation of monocyte life cycle will support arteriogenesis (rapid growth of preexisting collateral arteries).

Methods and Results: The continuous infusion of GM-CSF for 7 days into the proximal stump of the acutely occluded femoral artery of rabbits produced a marked arteriogenic response as demonstrated by an increase (2-fold) in number and size of collateral arteries on postmortem angiograms and by the increase of maximal blood flow during vasodilation measured in vivo (5-fold). When GM-CSF and MCP-1 were simultaneously infused the effects on arteriogenesis were additive. GM-CSF was also able to widen the time window of MCP-1 activity: MCP-1 treatment alone was ineffective when given after the third week following occlusion. When administered together with GM-CSF, about 80% of normal maximal conductance of was achieved. In vitro experiments showed that monocyte apoptosis was markedly reduced under GM-CSF treatment. In contrast to MCP-1, GM-CSF showed no activity on monocyte transmigration and also no influence on monocyte adhesion to cultured endothelial cells.

Conclusions: We have discovered a new function of GM-CSF as a powerful arteriogenic peptide. As a potential working mechanism it is proposed that it acts via prolongation of the life cycle of monocytes/macrophages.
GM-CSF, ACTING BY AMPLIFICATION OF MONOCYTE FUNCTION

Introduction

Circulating monocytes play a central role in both innate and acquired immunity of the host. Besides their crucial role in the defense against invading pathogens and a variety of other functions, monocytes play an obligatory role in adaptive growth and tissue remodeling of newly recruited collateral arteries (arteriogenesis)\(^1\)-\(^8\). However, the acquisition of functional competence and the ability to respond to a variety of activating or modulating signals requires maturation and differentiation of circulating monocytes to macrophages which in turn undergo several biochemical and phenotypic changes\(^9\). Importantly, the process of monocyte activation also confers survival signals essential for the functional integrity of these cells. Under the influence of chemotax attractants such as TNF-alpha and MCP-1, patrolling monocytes can be effectively recruited from the circulation to local inflammatory sites by attachment to the endothelium\(^10\)-\(^11\). Normally, these monocytes spontaneously undergo programmed cell death unless given "permission" to survive by special growth factors\(^12\)-\(^13\). This indicates that apoptosis may function as a major mechanism for reducing acute inflammation by elimination of unwanted cellular responses. During transendothelial migration, endothelial fas-ligand (fas-L) is one of the first important regulators that inhibits monocyte extravasation already during rolling and adhesion by inducing apoptosis via the monocytic Fas-receptor\(^14\)-\(^17\).

Circulating monocytes have a relatively short life span\(^18\) and their numbers are maintained by continuous replenishment from hemopoietic stem cells which in turn are dependent on the presence of a variety of accessory stromal cells including fibroblasts, macrophages, adipocytes, and endothelial cells in the bone marrow. Together with some of the mature blood cells, these cells are responsible for the production and presentation of a complex array of biologically active proteins (in particular Colony-Stimulating-Factors [CSFs]) which influence the development of blood cells. Granulocyte-Macrophage CSF (GM-CSF) enhances the survival\(^19\), proliferation\(^20\) and rate of differentiation\(^21\) of separate hemopoietic cell populations. Therefore, these substances are clinically used to treat patients with hematologic and oncologic disorders\(^22\)-\(^23\). Furthermore, several studies have demonstrated that CSFs influence lipid metabolism. They lower plasma cholesterol levels in humans, primates, and hypercholesterolemic rabbits by enhancing the clearance of LDL through both LDL-receptor-dependent and independent pathways\(^24\)-\(^26\). In addition, athromatous lesions in the aortic arch of Watanabe Heritable Hyperlipidemic Rabbits (WHHL) treated with human Macrophage-CSF (M-CSF) were significantly reduced as compared to normal control rabbits, indicating that CSFs prevented the progression of atherosclerosis\(^24\)-\(^26\)-\(^31\). We have previously shown that adhesion, activation, and migration of monocytes play an important role in collateral artery growth\(^1\),\(^4\)-\(^6\). After ligation of the arteria femoralis in the rabbit, shear forces in preexisting collateral arteries increase significantly, which leads to the upregulation of cell adhesion molecules (e.g., ICAM-1)\(^2\) and endothelial cytokine production such as MCP-1 and GM-CSF. These factors combine to effectively recruit circulating cells, in particular monocytes, to the sites of collateral artery growth. The monocytes itself mature into macrophages which produce large amounts of growth factors (MCP-1, b-FGF\(^1\)) as well as degratory enzymes such as
metalloproteinases. These factors in turn create an inflammatory environment which is necessary to build an artery from an arteriole. Increased activation and attraction of circulating monocytes via intraarterial infusion of MCP-1 into the collateral circulation can significantly influence this process of adaptive muscular collateral artery growth (arteriogenesis). In contrast, functional blockage of ICAM-1-dependent adhesion and transmigration of circulating cells (e.g., monocytes) via infusion of monoclonal antibodies against ICAM-1 in vivo significantly reduces collateral artery growth, thereby supporting the hypothesis that circulating cells dependent on ICAM-1 for transmigration are obligatory mediators of the arterial changes seen with MCP-1.

Since GM-CSF reduces monocyte apoptosis and is upregulated in endothelial cells under increasing shear stress, we have now tested the hypothesis that locally delivered GM-CSF is capable of promoting collateral artery growth by a direct effect on the rate of apoptosis of circulating and adhering monocytes as well as newly-recruited macrophages.

Materials and methods

Cytokine
Recombinant human GM-CSF synthesized by Escherichia coli was purchased from Novartis Pharma GmbH (Nürnberg, Germany).

Animal model
The present study was performed with the permission of the State of Hessen, Regierungspräsidium Darmstadt, according to section 8 of the German Law for the Protection of Animals. It conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985). Group 1 (1 week intraarterial cytokine treatment following femoral artery occlusion): For hemodynamic measurements four groups (each n=6) of NZWR received following infusions into the collateral circulation: GM-CSF (100 µg/day; 10 µL/h); MCP-1 (0.5 µg/day), the combination of both or solvent. Group 2 (1 week intraarterial cytokine treatment three weeks after occlusion) 24 NZWR were subjected to 21 days of right femoral artery occlusion. At this time the collateralization of these animals was completed before a growth factor treatment was started. After 21 days the animals were randomly assigned to identical treatments like in group 1. 7 or 21 days after pump implantation hemodynamic parameters were obtained: For the initial implantation of the osmotic minipumps, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (40 to 80 mg/kg body weight) and xylazine (8 to 9 mg/kg body weight). Supplementary doses of anesthetic (10% to 20% of the initial dose) were given intravenously as needed. The surgical procedure was performed under sterile conditions. Femoral arteries were exposed and cannulated with a sterile polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm) pointing upstream, with the tip of the catheter positioned distal to the branching of the arteria circumflexa femoris and the femoral artery distal to the catheter insertion site was ligated. The catheter was connected to an osmotic minipump (2ML-1, Alza 94)
GM-CSF, ACTING BY AMPLIFICATION OF MONOCYTE FUNCTION

Corporation, Palo Alto, CA), which was implanted under the skin of the lower right abdomen. The absence of any residual pump volume (<3%) after the experiment verified delivery of the contents. After closure of the incision and subcutaneous application of antibiotics, the animals were outfitted with plastic collars that allowed them to move freely but prevented self-mutilation. The rabbits were housed individually with free access to water and chow to secure mobility. Seven days after implantation the animals were again anesthetized with an intramuscular injection of ketamine hydrochloride and xylazine for tracheostomy and artificial ventilation. Anesthesia was deepened with pentobarbital (12 mg/kg body weight per hour). The carotid artery was cannulated for continuous pressure monitoring. The arteria saphena magna which corresponds to the anterior tibial artery in humans and is the main arterial supply to the lower limb and foot in the rabbit, was exposed just above the ankle and cannulated with sterile polyethylene heparinized tubing. These tubings were connected to a Statham P23DC pressure transducer (Statham, Spectramed) for measurement of peripheral pressures (PP). After heparinization with 5000 Units heparin, the left femoral artery was exposed and cannulated with sterile polyethylene catheter for the microsphere reference sample. After cannulation of the abdominal aorta a pump-driven shunt between the arteria carotis and the distal aorta was installed to perfuse both hindlimbs. A flow probe was installed to measure total flow to both hindlimbs.

In vivo Pressure-Flow Relations
Hemodynamic measurements and calculations of collateral conductance were performed as previously described. In brief, after the treatment period animals were anesthetized, heparinized and a pump-driven, flow controlled shunt between the carotid artery and the distal abdominal aorta was installed. Six differently labeled fluorescent microspheres (diameter 15 um; Molecular Probes) were injected into the shunt system, each at a different pressure level. To guarantee maximum vasodilatation, adenosine was continuously infused at a rate of 1 mg/kg/min. Peripheral and systemic pressures as well as total flow were measured and archived via a computer-based recording system (MacLab, MacIntosh). A reference sample was withdrawn at each pressure level. After digestion of muscle tissue samples and FACS-analysis for counting of microspheres, collateral conductance was calculated from the slope of the flow/pressure relations.

Postmortem angiography
X-ray angiograms were performed as previously described. Following Longland’s definition, only vessels showing a defined stem, midzone and re-entry, identifying them as collateral arteries, were counted.
CHAPTER 6

Detection of apoptotic monocytes

To evaluate the apoptosis rate of circulating monocytes we infused either Albumin (n=3), MCP-1 (n=3) or GM-CSF (n=3) or the combination of both (n=3) via osmotic minipumps into the collateral circulation of the rabbit hindlimb. During this time period (7 days) blood samples from each animal were obtained. Rabbit peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia & Upjohn, Freiburg, FRG). Monocytes were determined by FACS analysis via CD14 antigen expression and binding of FITC-conjugated Annexin-V (Alexis Corporation, NY) was used for detection of apoptotic cells. An anti-annexin-V-antibody (Alexis Corporation, NY) conjugated with FITC was used for detection of apoptotic cells. The anticoagulant annexin V is a member of a family of structurally related proteins that exhibit Ca$^{2+}$-dependent phospholipid binding properties. Annexin V binds to various phospholipid species with highest affinity for phosphatidylserine [PS]. In normal cells, PS is situated on the inner leaflet of the plasma membrane. When programmed cell death occurs, PS is translocated to the outer layer of the membrane, i.e. the cell surface. This occurs in the early phases of apoptosis during which the cell membrane itself remains intact. Furthermore apoptosis was confirmed by Hoechst-33342 staining and light microscopy (see below).

Analysis of monocyte apoptosis by fluorescence microscopy

Fluorescent DNA-binding dyes were used to define nuclear chromatin morphology as a quantitative index of apoptosis. Cells to be analyzed were stained with Hoechst 33342 (5 μg/ml), added to the culture medium for 20 min, at 37 °C. The media and the PBS rinses were collected and the cells were trypsinized. Media, PBS, and trypsinized cells were pooled and collected by centrifugation at 1200 rpm for 5 min at 4 °C. Cell pellets were resuspended in a small volume (50 μl) of serum-containing medium with 1 μg/ml Hoechst 33342 and 5 μg/ml Propidium iodide [PI]. An aliquot (25 μl) was placed on a glass slide, covered with a glass coverslip, and viewed under fluorescence microscopy. Individual nuclei were visualized at 400x to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells.

Transmigration assay

In order to rule out that GM-CSF promotes arteriogenesis via chemoattraction of monocytes, we tested transmigration of monocytes through Human Umbilical Venous Endothelial Cells (HUVECs). HUVECs were cultured in endothelial cell medium (Promocell, Heidelberg, Germany) to confluence on Millicell polycarbonate membranes (poresize 3 μm; Millipore, Eschborn, Germany). Confluence was observed by HE-staining. For performing transmigration MCP-1 and/or GM–CSF was diluted in macrophage serumfree medium and placed into the lower compartment. Isolated monocytes were diluted in Macrophage serumfree medium to a concentration of 10^6 cells/ml and placed into the upper compartment. After an incubation period of 3 hours the membranes including the upper compartment were removed and transmigrated cells were further cultivated for 12 h.
To analyze transmigration performance migrated cells were counted in a CASY 1 (Schaerfe Systems, Reutlingen, Germany) and apoptosis was quantified by FACS analysis using FITC-conjugated Annexin V (Alexis, Switzerland).

**Adhesion assay**
HUVECs were cultured to confluence on microtiterplates and stimulated for 4 h with TNF-alpha 10ng/ml; Sigma, Deisenhofen, Germany). The culture medium was removed and stimulated monocytes were incubated in macrophage serumfree medium (10⁴ cells/well) for 1 h. After this period the supernatant was removed and the microtiterplate was washed three times with PBS (pH 7.2). Adherent monocytes were then counted. Results are given as adherent monocytes per well.

**Evaluation of Apoptosis of adhering monocytes**
**FACS-Analysis:** Human isolated monocytes were allowed to attach to HUVECs (see above). Afterwards microtiterplates were carefully washed with PBS to remove non-adherent monocytes. 12 hours later, adherent monocytes were mobilized from the endothelial layer (15 minutes at 4°C) and immediately transferred to FACS-Analysis (CD 14 positive cells were gated and quantified for apoptosis staining [Annexin-V FITC]).

**Histological Analysis:** Monocytes attached to HUVECs were stained with antibodies against CD68, endothelial cells were stained with antibodies against CD 34. Apoptotic cells were stained according to the Tunel-protocol.

**Statistical Analysis**
Data are described as mean ± SD. Differences among data were assessed using unpaired Student’s t-test for intergroup comparisons and Mann-Whitney rank-sum test for unequal variances. Values of p ≤ .05 were required for assumption of statistical significance.

**Results**
In agreement with observations by Schaub et al.²⁹ our testing of blood samples (FACS) and blood smears (Cytology) confirmed that NZWR are "non responders": There was no significant increase in the number of circulating granulocytes and monocytes due to the GM-CSF treatment (Table 1).

**In vivo pressure-flow relations**
**Group 1:** early treatment (see above). Collateral conductance was significantly higher after 7 days of occlusion in animals treated with GM-CSF as compared to control animals (Figure 1). GM-CSF plus MCP-1 treatment showed an increase in collateral conductance of more than 40% (compared to maximal perfusion without ligation of the femoral artery). These values could not be reached by maximal MCP-1 application alone (≥ 8-10µg/day). The highest conductance levels after single-treatment with MCP-1 was 33 conductance units. **Group 2:** late treatment (see above). 7 day treatment with GM-CSF or MCP-1 in the fourth week after
Figure 1: Collateral Conductance after treatment with MCP-1, GM-CSF, combination of MCP-1 plus GM-CSF as compared to normal perfusion.

Ligation of femoral artery for 1 week, during this time period continuous infusion of solvent, GM-CSF, MCP-1 or the combination of MCP-1 plus GM-CSF.

Ligation of femoral artery for 3 weeks, in the fourth week continuous infusion of solvent, GM-CSF, MCP-1 or the combination of MCP-1 plus GM-CSF.

<table>
<thead>
<tr>
<th>Peripheral Blood</th>
<th>Solvent-Group</th>
<th>GM-CSF-Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td>4.1 ± 0.8 %</td>
<td>3.8 ± 1.0 %</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>35 ± 12 %</td>
<td>36 ± 9 %</td>
</tr>
</tbody>
</table>

Table 1
GM-CSF, ACTING BY AMPLIFICATION OF MONOCYTE FUNCTION

Figure 2: Postmortem Angiograms taken after 7 days of continuous infusion of solvent (A), GM-CSF (B) or MCP-1 (C). The stem (s), midzone (m) and reentry (r) can be clearly identified.

<table>
<thead>
<tr>
<th></th>
<th>solvent</th>
<th>GM-CSF</th>
<th>MCP-1</th>
<th>GM-CSF + MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of collateral arteries</td>
<td>15.4 ± 2.2</td>
<td>25.6 ± 3.6</td>
<td>30.1 ± 3.3</td>
<td>34.6 ± 2.1</td>
</tr>
</tbody>
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Table 2: number of visible collateral arteries (* indicates a p-value less than 0.05)

Figure 3: Number of monocytes adhering to HUVECs

Figure 4: Number of transmigrated monocytes
Figure 5: GM-CSF inhibits apoptosis of monocytes. A: Quantification of monocyte apoptosis via FACS analysis (Annexin V binding); B: dot plotting of apoptotic monocytes (FACS). C: Quantification of apoptosis via fluorescence microscopy. D: percentage of apoptosis if adhering monocytes.
GM-CSF, ACTING BY AMPLIFICATION OF MONOCYTE FUNCTION

Ligation is not sufficient to significantly improve arteriogenesis. In contrast, the concomitant application of MCP-1 plus GM-CSF enhances arteriogenesis to values of about 70% of normal maximal perfusion values.

**Radiographic findings**

Angiograms taken from hindlimbs of animals treated with GM-CSF from Group 1 (Figure 2b) as well as MCP-1 (Group 1) (Figure 2c) showed a remarkable increase in the number (Table 2), diameter and density of these collateral vessels as compared to animals with vehicle treatment (Figure 2a). The typical morphology of collateral arteries can be clearly identified: (stem [s], midzone [m], reentry [r], L= site of femoral artery ligation).

**Evaluation of monocyte adhesion**

Average adherence of untreated human monocytes to HUVECs was low (20 cells/well) and comparable to GM-CSF primed monocytes (44 cells/well). In contrast, monocyte adhesion after incubation with MCP-1 increased six fold to 110 adhered cells/well (Figure 3), which could not further be enhanced by concomitant GM-CSF application. While the number of adhering monocytes in the GM-CSF plus MCP-1 group was comparable to the single MCP-1 treatment, the percentage of protection from apoptosis was significantly higher when MCP-1 treated monocytes were also being pretreated with GM-CSF.

**Evaluation of monocyte transmigration**

The use of MCP-1 led to a significant increase in monocyte transmigration (9.2 x 10⁴ cells/ml) whereas GM-CSF* did not induce monocyte transmigration (comparable to untreated monocytes ° (1.7 x 10⁴ cells/ml vs. 1.6 x 10⁴ cells/ml) (Figure 4). Concomitant application of GM-CSF with MCP-1 did also not increase the number of transmigrated monocytes.

**Detection of monocyte apoptosis via light microscopy and FACS analysis**

In the GM-CSF treated monocyte group the rate of apoptosis was significantly reduced. Annexin V-dependent fluorescence decreased from a mean value of 34.68 in the control group to 14.08 in the GM-CSF-treated group. Additionally, the GM-CSF-treated group also showed a significantly lower apoptosis rate when compared to a MCP-1 treated group (mean fluorescence: 24.5) (Figure 5a and b). This was confirmed via qualitative fluorescence microscopy of Hoechst-33342-stained cells (Figure 5c and d). Apoptotic monocytes showed the characteristically modified nuclei (Figure 6a). FACS-Isolated Annexin V positive monocytes showed typical patterns of apoptosis (Figure 6b) as compared to non-apoptotic monocytes (Figure 6c). In the macrophage group a similar reduction of apoptosis after GM-CSF treatment was observed via FACS-Analysis. Figure 6f shows the typical morphology of a non-apoptotic macrophage (electron microscopy sections kindly provided by Dr. Keisuke Suzuki). The nuclear pattern of this macrophage is not condensed, the cell exhibits large amounts of endoplasmic reticulum, indicating
Fig 6: A: Nuclear chromatin morphology of monocytes was analyzed via a quantitative index of apoptosis (Hoechst 33342 and Propidium iodid staining). Individual nuclei were visualized at 400x to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells (arrow). B: Apoptotic monocyte with margination and condensation of chromatin, fragmented nuclei and intact cell membrane. C: Nuclear pattern of non-apoptotic monocytes. The cells show no condensed chromatin. D: Nuclear pattern of non-apoptotic macrophage. This macrophage possesses a large amount of endoplasmic reticulum, indicating its activity in protein synthesis (electron microscopy: x 5000, bar=mm). E: Apoptotic macrophage with margination and condensation of chromatin, fragmented nuclei and intact cell membrane (electron microscopy: x 5000, bar=mm). The endoplasmic reticulum is only weakly visible. F: In-vitro adhesion assay: Double Staining for adhering macrophages with CD 68 (m), endothelial cells (e) and apoptosis after the Tunel-protocol (m°). G: In-vitro adhesion assay: Magnification of an apoptotic monocyte.
high protein turnover. Figure 6g (electron microscopy section) shows an apoptotic macrophage with margination and condensation of chromatin and fragmented nuclei. Morphological signs of cell activity are only weakly visible (endoplasmic reticulum) while the cell membrane is intact.

**Histological analysis of monocyte apoptosis during adhesion**

Fig. 6d shows monocytes attaching to confluent HUVECs. After attachment microtiterplates were carefully washed with PBS. Adherent monocytes can be identified with antibodies against CD68 (red). Apoptotic monocytes exhibit a condensed pattern of chromatin. These monocytes were Tunel positive (m²). Endothelial cells can be identified in the background (6e).

**Discussion**

Circulating monocytes originate from pluripotent hemopoietic progenitors in the bone marrow and provide a broad spectrum of physiological and pathophysiological properties: Once they leave the bloodstream, fractions of monocytes serve as precursors for several other cells with phagocytic function (Kupffer cells of liver, osteoclasts of bone, etc.) or antigen presenting dendritic cells. Others mature into tissue macrophages and are responsible for removal of debris as well as defense against invaders such as fungi and bacteria, which cannot be dealt with effectively by neutrophils (unlike neutrophils, macrophages are able to regenerate their lysosomal granules and may thus have a longer lifespan than neutrophils). However flow cytometry and functional monocyte assays have shown that monocytes are a very heterogenous group of cells: Only 30-40% respond to chemoattractants, the expression of α and β-integrins and of the cell-surface HLA-DR antigen varies significantly. The capacity to produce reactive oxygen species is very different suggesting that only subpopulations of monocytes are able to participate in specific immune responses. Moreover it has recently been reported that a subset of CD34- monocytes contains a fraction with the potential to differentiate into an endothelial phenotype. Despite this heterogeneity all monocytes derive from common hematopoietic precursor cells and require a complex array of biologically active proteins (in particular CSFs) which influence their development and survival.

The main findings of our study are that chronic intra-arterial infusion of GM-CSF stimulates the development of arterial collateral blood vessels (arteriogenesis) following femoral artery occlusion. These are more numerous on angiograms and their ability to conduct blood had increased by a factor of 5-fold. The mechanism of action is the prolonged survival of monocytes-macrophages, known to play a decisive role in arteriogenesis. Furthermore GM-CSF is a powerful adjunct to the treatment with CC-Chemokines (MCP-1) to induce arteriogenesis. The highest value of collateral conductance after 1-week high-dose MCP-1 alone treatment reached 20% of normal perfusion values whereas the combination therapy of MCP-1 plus GM-CSF was twice effective (conductance more than 42% of normal perfusion values). For the exogenous supply of several angiogenic growth factors only a brief time window is available for action, usually within hours or days following arterial occlusion.
occlusion. This limits their therapeutic power in subjects with a stable but functionally deficient collateral circulation. Therefore we tested whether growth of collateral arteries can be re-started after long term femoral ligation in the rabbit. Three weeks after ligation of the femoral artery both MCP-1 as well as GM-CSF were infused for one week into the matured collateral circulation. Both single treatments did not improve perfusion markers as compared to the natural course of collateral artery growth. However when GM-CSF and MCP-1 were infused simultaneously the effects on arteriogenesis were additive on angiograms as well as on conductance. The effect of the combined treatment at four weeks after femoral occlusion was about 80% of normal maximal conductance of the artery that was replaced by collaterals, a result that was not reached before by any other experimental treatment.

In previous studies we had shown that stimulation of monocyte function by LPS and by infusion of the monocyte chemoattractant factor MCP-1 greatly increased arteriogenesis. Arteriogenesis differs from angiogenesis in several aspects, the most important being the dependence of angiongenesis on hypoxia and the dependence of arteriogenesis on inflammation. Collateral arteries grow surrounded by tissue that is not ischemic: its resting blood flow is not decreased, its ATP and PCR content is normal and hypoxia-induced gene transcription (LDH-A, VEGF) is not activated. Arteriogenesis is by far the most efficient adaptive mechanism for the survival of ischemic limbs or internal organs like the heart because of its ability to conduct, after adaptive growth, relatively large blood volumes per unit of time. An increased number of capillaries, the result of stimulated angiogenesis, is unable to do that.

The inflammatory environment which is necessary for arteriogenesis is created by homing of circulating cells to shear stress activated endothelium which express MCP-1. These circulating cells are mainly monocytes (derived from hemopoietic stem cells) and lymphocytes but also basophiles that transform in the tissue into mast cells where they produce vasoactive substances.

Our present study supports the inflammatory paradigm of arteriogenesis because GM-CSF prolongs the duty cycle of monocytes-macrophages and protects high numbers of MCP-1 attracted monocytes from apoptosis and thereby enhances their arteriogenic activity. CSFs are essential for the survival of macrophages and significantly influence the rate of apoptosis of circulating and adherent blood monocytes as well as resident tissue macrophages. Monocytes circulate in the blood for 12-72 hours. Those cells that are attracted to the site of inflammation by chemotactic factors will nonetheless die by apoptosis unless provided with specific survival signals. It is important to recognize that programmed cell death occurs without inflammation and is a normal physiological response by many eukaryotic cells to factors and conditions that are, as yet, poorly defined. In the normal resolution of an acute inflammatory response apoptosis of monocytes and neutrophils is essential to maintain immune homeostasis and limit inappropriate host tissue damage by decreasing white blood cell tissue load, function, and release of phlogistic reactive oxygen species and proteases. Monocytes are known to undergo spontaneous apoptosis upon leaving the circulation unless provided with specific survival signals. Interestingly, factors that are needed for the recruitment of
monocytes into inflammatory lesion, namely TGF-1, bacterial peptides and MCP-1 have little or no effect on monocyte survival\textsuperscript{18}.

Taken together these data are consistent with our observations. The increase of shear stress initiates the recruitment of monocytes by upregulation of adhesion molecule expression on the endothelial lumen of the collateral vessel. Simultaneously the production of GM-CSF by the endothelium is rapidly increased\textsuperscript{33}. Since the chemoattractive action of GM-CSF on monocytes is rather weak the monocyte recruitment is mediated by a direct effect of GM-CSF on monocyte activation, proliferation, differentiation and motility, and secondarily, by chemoattractant molecules released in response to the locally administered GM-CSF. The present in vivo study also indicates that GM-CSF is able to significantly prolong the life-span of monocytes in vivo via a reduction of apoptosis. These data were confirmed by our in vitro studies, showing the protective effect of GM-CSF on monocytes and macrophages. The demonstration of a potent therapeutic effect of exogenously administered GM-CSF on collateral artery growth in this model confirms our previous reports about the central role of monocytes during the rapid growth of muscular collateral arteries (arteriogenesis). Since CSFs have remarkable anti-atherogenic effects\textsuperscript{24,26,31}, GM-CSF might provide an important mechanism, other than chemoattraction\textsuperscript{18}, to significantly enhance arteriogenesis in atherosclerotic subjects. It is worth noting that macrophages can in principle be their own source of regulatory cytokines such as GM-CSF which in turn upregulate their own production of cytokines\textsuperscript{18}. Since this mechanism is clearly suboptimal, substitution therapy, as in our study, is more effective. Finally exogenous GM-CSF may also release hemopoietic stem cells from the bone marrow that may be even more potent stimulators of arteriogenesis than circulating monocytes as our preliminary studies imply (in preparation).
CHAPTER 6

References


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