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Pro-angiogenic induction of myeloid cells for therapeutic angiogenesis can induce mitogen-activated protein kinase p38-dependent foam cell formation

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Abstract

Background aims—Clinical trials for therapeutic angiogenesis use blood- or bone marrow-derived hematopoietic cells, endothelial progenitor cells (EPC) and mesenchymal stromal cells (MSC) for vascular regeneration. Recently concerns have emerged that all three cell types could also contribute to atherosclerosis by foam cell formation. Therefore, we asked whether human myelomonocytic cells, EPC or MSC can accumulate lipid droplets (LD) and develop into foam cells.

Methods—LD accumulation was quantified by flow cytometry, confocal microscopy and cholesterol measurement in each of the cell types. The impact of an initial pro-angiogenic induction on subsequent foam cell formation was studied to mimic relevant settings already used in clinical trials. The phosphorylation state of intracellular signaling molecules in response to the pro-angiogenic stimulation was determined to delineate the operative mechanisms and establish a basis for interventional strategies.

Results—Foam cells were formed by monocytes but not by EPC or MSC after pro-angiogenic induction. Mitogen-activated protein kinase (MAPK) p38 phosphorylation was enhanced and kinase inhibition almost abrogated intracellular LD accumulation in monocytes.

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Supplementary material available online Figures S1–S4.

Conclusions—These data suggest that hematopoietic cell preparations containing monocytes bear the risk of foam cell formation after pro-angiogenic induction. Instead, EPC and MSC may drive vascular regeneration without atherogenesis aggravation. A thorough understanding of cell biology is necessary to develop new strategies combining pro-angiogenic and anti-atherogenic effects during cell therapy.

Keywords

atherogenesis; foam cell formation; mitogen-activated protein kinase inhibition; stem cell therapy; therapeutic angiogenesis

Introduction

Therapeutic angiogenesis by stem cell transplantation was initially designed to reconstitute tissue vascularization and promote cardiovascular repair. Angiogenesis shares several features with atherogenesis, indicating that ‘interrelated trade-offs’ might be inherent in vascular regenerative cellular therapy (1). The pathophysiology of atherosclerosis is initiated by the adhesion of immune cells to the dysfunctional endothelial surface, their transmigration and differentiation into foam cells or pro-inflammatory lymphocytes. These key events trigger the development of atherosclerotic plaques, with enhanced proliferation of smooth muscle progenitors forming a cap around necrotic lipid deposition within the vascular walls. Chronic inflammation, subsequent plaque instability and sustained lipid deposition in increasing plaques via the recruitment of monocyte-derived lipid-enriched foam cells are considered to be hallmarks of atherosclerosis. The double-edged role of monocytic cells in this scenario is now increasingly recognized: The cross-talk with other hematopoietic or vascular progenitor cells may result in either collateralogenesis/angiogenesis or atherogenesis (2). A considerable body of experimental and clinical data indicates that tissue repair is promoted via pro-angiogenic stimuli of human blood- or bone marrow (BM)-derived cells, with a phenotypic overlap with endothelial progenitor cells (EPC) (3–7). Controversial data have been reported from animal experiments indicating accelerated atheroma progression in addition to vascular regenerative stimuli after treatment with stem cells derived from the BM mononuclear cell (MNC) fraction (8,9). Despite improved cardiac function, an unexpectedly high incidence of restenosis, increased plaque burden and plaque inflammation in the infarct-related vessels has been reported in clinical studies exploring the effects of injected BM stem cells or mobilized peripheral blood (PB)-derived cells after myocardial infarction (10–12).

Most therapeutic angiogenesis studies have considered EPC to represent the cellular candidates of interest for vascular regenerative therapy within the transplant. In addition to EPC, transplants derived from human blood and BM also contain sizable numbers of hematopoietic cells, including monocytes/macrophages and non-hematopoietic mesenchymal stromal cells (MSC) that can function as pericyte and smooth muscle progenitors. As each of these cell types may be involved in atherogenesis, their transition into lipid-storing foam cells could indicate one potential mechanism leading to harmful side-effects of therapeutic angiogenesis, as observed in clinical and experimental studies (13–15). This may be even more relevant if pro-atherogenic pathologic conditions at the time of treatment are sustained. Over the last few years, we and others have developed procedures to isolate and propagate hematopoietic, endothelial and mesenchymal precursors for therapeutic application (16–22). Therapy with specifically manipulated cells to promote tissue vascularization and repair after organ damage is a central goal of regenerative medicine. Pro-angiogenic growth factors have been used to induce an angiogenic switch within autologous stem and progenitor cells prior to re-application (23,24). In this study we examined the influence of pro-angiogenic conditioning on the foam cell formation of human

blood- and BM-derived cells as a potential indicator for pro-atherogenic side-effects of therapeutic angiogenesis.

Methods

Generation of human monocytes, EPC and MSC, and pro-angiogenic culture

CD14⁺ monocytes were isolated from PB MNCs from healthy donors using MACS technology (Miltenyi, Bergisch Gladbach, Germany). CD14 purity was checked by flow cytometry (supplementary Figure S1). BM-derived MSC were isolated in alpha-modified Eagle's medium (α -MEM; Sigma-Aldrich, St Louis, MO, USA) via plastic adherence (17,18). Primary EPC, also called endothelial colony-forming progenitor cells (ECFC), were directly recovered from heparinized but otherwise unmanipulated PB by plastic adherence in endothelial growth medium (EGM-2; Lonza, Walkersville, MD, USA) (19).

Initial plating density was $3.5\text{--}4.7 \times 10^5$ CD14⁺ sorted monocytes/cm². To expand MSC and EPC, $4.6\text{--}22.3 \times 10^3$ /cm² BM-derived MNC/cm² and $2.5\text{--}6.6 \times 10^5$ /cm² PB-derived nucleated cells were seeded. The initial colony frequency for MSC was 1/1546–1/921 and for EPC 1/2 500 000–1/1 000 000. Primary monocytes, early passage EPC and MSC (after a maximum of 8–10 population doublings) were incubated separately in pro-angiogenic endothelial growth medium (EGM) containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), heparin, hydrocortisone (HC), epidermal growth factor (EGF), insulin-like growth factor (IGF), gentamycin, amphotericine and 10% fetal bovine serum (FBS) (EGM-2). The study was approved by the institutional review board of the Medical University of Graz (Graz, Austria) (protocol number 19-252 ex 07/08). The investigation conformed to the principles outlined in the Declaration of Helsinki.

Assessment of foam cell potential

The experimental design to investigate the foam cell capacity of monocytes, EPC and MSC is shown in Figure 1. Cells were either exposed immediately to acetylated low-density lipoprotein (acLDL; 30 μ g/mL final concentration) or subjected to a precedent pro-angiogenic induction for 3 days in EGM-2 without acLDL. LDL was isolated from human plasma by density-gradient ultracentrifugation and acetylated as described elsewhere (25). A minimum of 12 h of acLDL exposure was carried out to permit cellular lipid uptake (Figure 1; test condition I, +12h/acLDL). Extended acLDL exposure was tested with daily supplementation over a period of 84 h (condition II, +84h/acLDL). The impact of an initial 3-day pro-angiogenic induction in EGM-2 before acLDL exposure on foam cell formation was studied in a third group (condition III, pro-angiogenic induction + 12h/acLDL, incubation time 72 + 12 h). Finally, the cumulative effect of the pro-angiogenic induction with a prolonged lipid exposure was tested (condition IV, pro-angiogenic induction + 84h/acLDL, incubation time 72 + 84 h). Control cultures lacking acLDL supplementation were also performed.

Intracellular lipid detection by confocal microscopy and flow cytometry

Intracellular accumulation of neutral lipids within lipid droplets (LD) was determined morphologically after Nile Red staining (Sigma-Aldrich; 0.15 μ g/mL final concentration) with a confocal laser scanning microscope (CLSM; LSM 510 Meta, Axiovert 200M; Zeiss, Jena, Germany). Vertical 0.5- μ m sections resulting in 10–30 virtual slices were recorded (Zeiss LSM image browser version 3.5.0.359). To analyze at least 40–100 cells per test condition, virtual Z-stacks of 4–10 high-power fields were explored. Fluorescence signals were detected using a 543-nm laser and a 560-nm long pass filter and the detector gain was set at 799. ImageJ® software v1.37 (<http://rsbweb.nih.gov>, accessed date: October 2009) was used for standardized quantification of LD/cell. To this end, photomicrographs of each

high-power field were processed to depict LD only and an automated LD count was done using a pixel size threshold set once for all analyzes. The events counted as LD were recorded for each particular level within the analyzed cells (count strategy is illustrated in Figure 2C). This method allowed for a reliable comparison of the LD accumulation characterizing the foam cell potential in the different cell types after the indicated culture conditions. The resulting arbitrary units (AU) reflected the relative LD amount per cell. LD larger than 0.5 μm could be counted in more than one virtual section.

The Nile Red reactivity of neutral lipids per single cell was detected using flow cytometry in a Phycoerythrin (PE) channel [FL-2 detector, equipped with a 585/42 bandpass filter; FACSCalibur®; Becton Dickinson (BD), Erembodegem, Belgium]. IgG1–PE monoclonal antibodies at a final concentration of 2 $\mu\text{g}/\text{mL}$ were used as controls. The mean fluorescence intensity (MFI) of 20 000 viable monocytes, EPC and MSC treated with the defined test conditions was measured and compared with controls. Light scatter properties were determined as a measure of cell size and granularity.

Lipid quantification in monocytes, EPC and MSC using gas chromatography–mass spectrometry

Monocytes, EPC and MSC derived from three donors were cultured in six-well polystyrene plastic plates (BD) and harvested by trypsinization after the indicated treatments. Lipids were extracted using hexane/isopropanol (3/2 v/v, 1 h, 4°C, continuous agitation, $n = 3$). To each sample 5- α -cholestane (0.55 mg/dL final concentration) was added as an internal standard. The content of total cholesterol was quantified using a dual-stage Quadrupole gas chromatography–mass spectrometry (GC-MS) instrument (Thermo Fisher, Palo Alto, CA, USA). Total cholesterol values are given as the area under the curve (AUC) normalized with the internal standard and corrected results are normalized to the respective protein concentrations (Figure 3B).

Intracellular protein phosphorylation state analyzes of human monocytes by flow cytometry

Monocytes were exposed to pro-angiogenic induction for 5 or 30 min at 37°C to determine the immediate and short-term effects of growth factor-containing EGM-2 on kinase phosphorylation. Controls were incubated with growth factor-free endothelial basal medium (EBM). Cell fixation was done using Cytofix and permeabilization buffer provided with the Phosflow kit (BD). Monoclonal antibodies detecting the phosphorylated forms of the mitogen-activated protein kinase p38 (MAPK p38, pT180/pY182), extracellular signal-regulated kinase (ERK1/2, p44/42 MAPK) and the signal transducers and activators of transcription STAT1 (pY701 and pY727) and STAT3 (pY705 and pS727; BD), were used for staining. IgG1–Alexa488 were used as control antibodies for MAPK p38, ERK1/2, STAT1 pY727 and STAT3 pS727. IgG2a–Alexa488 control antibodies were used for STAT1 pY701 and STAT3 pY705 expression analyzes. All monoclonal antibodies were used at 0.6 $\mu\text{g}/\text{mL}$ final concentration and staining was performed at room temperature for 25 min. Data were acquired using a FACSCalibur® instrument and evaluated with FlowJo software (FlowJo®, Ashland, OR, USA). Differences in MFI values from specific phosphokinase reactivities minus corresponding control values were calculated (ΔMFI ; Figure 4). To confirm the specific MAPK p38 inhibition, we performed experiments using the specific MAPK p38 inhibitor SB203580 compared with untreated monocytic cells or cells treated with dimethylsulfoxide (DMSO) only in EGM-2 medium.

Foam cell potential assessment with MAPK p38 inhibition

Monocytes were cultured with a 3-day pro-angiogenic induction phase and subsequently exposed to acLDL (according to conditions III and IV). The specific MAPK p38 inhibitor

SB203580 (10 μM ; Sigma-Aldrich) dissolved in DMSO (Wak-Chemie, Steinbach, Germany) was added either daily from the start of culture during pro-angiogenic induction (indicated as start-to-finish) or subsequently just before acLDL loading (pre-acLDL). Control cultures were incubated with DMSO (0.1% v/v). LD/cell was determined by CLSM after Nile Red staining.

Statistical analysis

SPSS 15.0 software (SPSS Inc., München, Germany) was used for the Wilcoxon test. Results are shown as the median with the first and third quartiles, and minimum/maximum values are depicted as whiskers in box plots. Significance was set at $P < 0.05$. Data sets with less than five samples are depicted with descriptive statistics showing mean values $\pm$ standard deviation (SD).

Results

Foam cells develop from human monocytes in vitro

Magnetically sorted monocytes (CD14 purity 97.9–99.3%; supplementary Figure S1), EPC and MSC containing less than 1% hematopoietic contamination (17,26,27) were tested for their foam cell potential. Short-term acLDL exposure for 12 h did not result in foam cell formation (test condition I, median 1.5 LD/cell; Figure 2A, row 1 and 2, and Figure 2B). Foam cells started to develop from monocytes within 84 h of acLDL exposure (condition II, 19 LD/cell) but LD accumulation was augmented within the same time frame if a 3-day pro-angiogenic induction preceded the 12 h of acLDL exposure (condition III, 41.5 LD/cell). The number and size of Nile Red-stained LD/cell further increased after pro-angiogenic induction plus an extended acLDL exposure (condition IV, 102 LD/cell) even though monocytes were not differentiated into macrophages using external macrophage-colony-stimulating factor (M-CSF) induction. In contrast, EPC and MSC entirely lacked LD accumulation in all lipid exposure conditions (Figure 2A, rows 3–6), as well as in control cultures (Figure 2A, row 7). Supplementary Figure S2 shows Z-stack movies after pro-angiogenic induction and 84 h of acLDL from each test cell type. The software-assisted LD counting strategy applied to all experiments is also shown in one representative sample after pro-angiogenic induction plus extended acLDL exposure of monocytes (condition IV; Figure 2C).

Cholesterol accumulates in human monocytes

Flow cytometry of monocytes, EPC and MSC confirmed that the amount of LD increased over time exclusively in acLDL-loaded monocytes. The Nile Red reactivity was higher following pro-angiogenic induction plus 84-h acLDL loading compared with the pro-angiogenic induction and short-term acLDL treatment for 12 h (MFI values of 26 ± 5 in condition IV versus 19 ± 4 in condition III; Figure 3A, top). In addition, high side-scatter properties (SSC) as a measure of the cellular granularity were found as a result of LD accumulation [top forward scatter/side scatter dot-plot (FSC/SCC), upper right histogram]. Control monocytes showed low SSC properties (bottom dot-plot, upper right histogram). The exposure to acLDL did not result in lipid accumulation in EPC or MSC, although EPC are commonly recognized by their capacity to take up LDL (Figure 3A, middle and bottom histograms). This finding was supported by low SSC properties of EPC and MSC, documenting the lack of intracellular granules in both the acLDL supplemented and control conditions (dot-plot inserts, right histograms, middle and bottom rows). Generally, macrophage-derived foam cells are defined biochemically by accumulated cholesteryl esters stored within LD to protect cells from unesterified cholesterol and free fatty acids (28). The GC-MS quantification of total cholesterol revealed a distinct increase within acLDL-loaded monocytes compared with controls. The highest total cholesterol amounts were found after

pro-angiogenic induction plus 84 h of acLDL exposure (condition IV; Figure 3B). GC-MS measurements were consistent with enhanced LD counts and high Nile Red reactivity found after this treatment but not after only 12 h of acLDL exposure (condition III). This may be because of the smaller size of LD at the earlier time-point. Detailed total cholesterol values per microgram protein for all three cell types are shown in supplementary Figure S3. These data revealed that pro-angiogenic induction followed by an extended acLDL exposure resulted in maximum amounts of total cholesterol within monocytes. EPC and MSC contained unchanged amounts of total cholesterol per microgram protein independent of acLDL presence.

MAPK p38 is activated in monocytes following pro-angiogenic induction

It has been shown that the MAPK p38 pathway is required for foam cell differentiation of macrophages exposed to oxidized LDL (29). Furthermore, MAPK p38 and other kinase cascades are involved in a broad range of inflammatory responses (30). We analyzed monocytes after a 5- or 30-min exposure to pro-angiogenic induction for immediate phosphorylation of intracellular signaling molecules by flow cytometry. We found a sustained activation of MAPK p38 in monocytes after a 30-min pro-angiogenic induction (Figure 4A, B). There were no differences in the phosphorylation state of MAPK p38 after 5 min and no differences in ERK1/2, STAT1 and STAT3 phosphorylation following 5- and 30-min incubations of monocytes in growth factor-supplemented complete EGM-2 (Figure 4C). Phosflow analyzes of monocyte preparations ($n = 3$) showed a clear immediate and early onset inhibition of MAPK p38 phosphorylation after SB203580 treatment that was not found in controls incubated in pro-angiogenic medium only or with additional DMSO (supplementary Figure S4).

Foam cell formation of monocytes is MAPK p38 regulated

The role of MAPK p38 in the process of LD accumulation after a 3-day pro-angiogenic induction of monocytes was evaluated in MAPK p38 inhibition experiments using the specific p38- α inhibitor SB203580. Nile Red staining of intracellular neutral lipids after pro-angiogenic induction and the indicated treatment is shown in Figure 5A. MAPK p38 inhibition with SB203580 almost abrogated LD accumulation in monocytes after 12 and 84 h of acLDL exposure. The LD reduction was independent of the time-course of MAPK p38 inhibition (Figure 5B).

Discussion

We have demonstrated that a 3-day pro-angiogenic induction renders human monocytes susceptible to foam cell formation under moderate lipid stress. To date, it has not been shown that primary monocytes bear the risk of foam cell development directly after pro-angiogenic induction. The culture conditions in our study mimic protocols that have been used to induce angiocompetence in BM-derived cells before intracoronary application in clinical trials (6,7,31,32). Notably, the acquisition of a distinct foamy appearance was independent of external M-CSF stimulation. This monocyte-to-foam cell induction occurred at earlier time-points and was induced by lower acLDL concentrations compared with previous studies reporting foam cell development without pro-angiogenic induction (33–35). MAPK p38 phosphorylation was rapidly up-regulated in primary monocytes after stimulation with angiogenic growth factor-containing medium. As foam cell formation *in vitro* was reduced by selective MAPK p38 inhibition, we consider the MAPK p38 pathway to be involved critically in lipid uptake mechanisms of pro-angiogenically primed monocytes. In line with this observation, it has been reported very recently that in a human monocytic cell line oxidized LDL also stimulates the expression of a pro-atherogenic phospholipase via MAPK p38 (36). The foam cell potential of myeloid cells has been

published repeatedly since the late 1970s and recent studies have further unraveled detrimental monocyte effects in the cardiovascular system (33–35).

The capacity for LDL uptake is an extensively discussed attribute of EPC, whereas the foam cell potential of fibroblasts and mesenchymal smooth muscle cell precursors from both humans and animals remains controversial (15,25,37,38). Given the degree of attention stem cell therapy for cardiovascular repair has attracted, it is interesting that key issues at the cellular level still remain uncertain. A major problem is that, despite being BM-derived, cells used in clinical trials mainly consist of mature blood cells and only minute portions of well-defined stem cells. Up to now, moderate benefits have been observed, particularly in randomized clinical trials (38–42). Studies focusing on side-effects initiated at the cellular level in the human setting are rare, despite the fact that results from animal studies have already sounded a note of caution (8,10–12,43). We investigated the susceptibility of blood- and BM-derived cells to lipid stress and the activation of signaling pathways under defined culture conditions. The *in vitro* acLDL concentration tested corresponded to 2–3% of the physiologic lipid stress exerted *in vivo* by LDL in humans (1000–1500 µg/mL) (39). Concentrations of acLDL corresponding to these 50-fold higher reference levels could not be applied *in vitro* because we and others have observed increasing cell damage after exposure to LDL levels exceeding 100 µg/mL (40). We did not find LD in MSC or EPC, although we and others have shown that EPC can be characterized by LDL uptake (5,15,37). Therefore, we speculate that lipoproteins entering EPC are not stored but simply cross the endothelial layer. However, it remains unclear whether these findings translate to the *in vivo* situation. It has been shown clearly that pro-angiogenic conditioning induces monocytes to form foam cells *in vitro*. It remains to be determined whether EPC or MSC can form foam cells after extended induction *in vitro* or *in vivo*. Atheroma aggravation *in vivo* has been shown in animal experiments and clinical trials investigating the effects of cellular therapeutic angiogenesis (8,9,11,12). These data support our assumptions regarding the double-edged role of cell therapy effects on the vascular system *in vivo*. Therefore, we argue further that cellular products containing significant amounts of stress-sensitive myelomonocytic cells may bear the risk of *de novo* atherogenesis or atheroma aggravation. This is exemplified by Mansour *et al.* (11) showing a substantial risk of ‘luminal loss, stent restenosis or *de novo* stenosis’ correlated with escalating doses in patients after BM-derived cellular therapy. In their study, these authors determined the plaque burden by intravascular ultrasound of the infarct-related arteries after intracoronary delivery of BM-derived cells (11). In a recent prospective randomized clinical study, the reported restenosis risk was similar in both the BM MNC-treated and placebo groups. It can be speculated that restenosis in that particular trial was inhibited additionally by the use of Paclitaxel®-coated stents for anti-proliferative prophylaxis (41). Another trial resulted in a high incidence of occlusion in the infarct-related arteries after injection of BM-derived progenitors with a 28% monocyte contamination (12). In contrast to these results, the effects of circulating myelomonocytic ‘angiocompetent’ cells were expected to promote therapeutic angiogenesis (42). Some studies provide evidence that paracrine factors secreted by myelomonocytic cells promote endothelial cell function and cardiovascular regeneration *in vitro* and *in vivo* (27,43). Recently, recruited myeloid cells have been shown to constitute important cellular components of early vasculogenic processes *in vivo* (39). The current discussion considers monocyte activation rather than monocyte suppression as a potential therapeutic target in cardiovascular diseases. Consequently, if foam cell formation of myeloid cells could be reduced through kinase inhibition, their combined pro-angiogenic but equally anti-atherogenic effects could offer novel therapeutic strategies. Our goal is to develop efficient and safe cell therapy protocols with the focus on blood- or BM-derived cells to promote angiogenesis in damaged organs and tissues. A thorough understanding of cellular mechanisms is necessary to augment further the potential benefits of cellular therapeutic angiogenesis. Thus combined angiocompetent effects of particularly engineered

hematopoietic cells that act synergistically with EPC and MSC could pave the way for novel strategies in cardiovascular stem cell therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental design: analyzing the foam cell potential of isolated human blood- or BM-derived cells. Monocytes, EPC and MSC were tested for their foam cell capacity with acLDL loading either immediately after isolation or following 3 days of pro-angiogenic induction.

Figure 2.

Monocytes but neither EPC nor MSC develop into foam cells after pro-angiogenic induction. (A) Intracellular accumulation of LD in monocytes, EPC and MSC was determined by phase-contrast and fluorescence microscopy after Nile Red staining. Four different culture conditions with or without a 3-day pro-angiogenic induction and 12 or 84 h of acLDL exposure were tested (I-IV). Corresponding control stainings without acLDL exposure are shown in the bottom panel. The length of the scale bars is 10 μ m (monocytes) and 20 μ m (EPC and MSC). (B) Enumeration of the intracellular LD per cell within different monocyte preparations revealed that pro-angiogenic induction before acLDL exposure increased foam cell formation *in vitro*. Data are shown in box-plots as medians with the first and third quartiles [q₁/q₃] as well as minimum and maximum values [min/max, whiskers], $n = 6$; + indicates an outlier. (C) A representative sample of monocytes after pro-angiogenic induction plus 84 h acLDL illustrates how LD were highlighted with ImageJ® software. In sum, 1328 LD were counted within 11 monocytes in 12 virtual 0.5- μ m sections along the z-axis, resulting in 120 LD/cell (AU). The LD count of each virtual section is shown in brackets.

Figure 3.

Lipid storage in myelomonocytic cells is enhanced after pro-angiogenic induction. (A) Blood monocytes, EPC and BM-derived MSC after the indicated treatments were analyzed using flow cytometry. Dark gray histograms show the Nile Red fluorescence intensity of acLDL-supplemented cell preparations compared with IgG1-PE controls (thin light gray). Inserts show FSC/SSC properties of acLDL-supplemented (top) and control monocytes (bottom dot-plot, right histogram, first row). EPC and MSC displayed low SSC properties because of a lack of intracellular droplet accumulation after acLDL-supplemented and control culture (dot-plots, right histograms, middle and bottom rows, $n = 4$). (B) Total cholesterol quantification of identical samples was done using GC-MS. Dark bars represent cultures with, and light gray bars without, acLDL loading. Results are shown as mean AUC values normalized to the protein concentrations $\pm$ SD ($n = 3$).

Figure 4.

MAPK p38 phosphorylation increases in monocytes after pro-angiogenic induction. (A) The phosphorylation of intracellular signaling molecules after monocyte contact with growth factors and hormones during pro-angiogenic induction was measured using phospho-specific antibodies by flow cytometry. (B) MAPK p38 phosphorylation was found to be reduced in monocytes after 30-min contact with unsupplemented basal medium (light gray, EBM) in comparison with sustained stimulation of monocytes with growth factor-containing (dark gray) complete EGM-2. Histograms with thin lines show reactivity with IgG1–Alexa488 control stainings. (C) No difference in ERK1/2p44/42, STAT1pY701, STAT1pY727, STAT3pY705 and STAT3pS727 phosphorylation was found between EBM and complete EGM-2 incubation ($n = 7$).

Figure 5.

Foam cell development is reduced after MAPK p38 inhibition. (A) Morphologic assessment after pro-angiogenic induction of monocytes exposed to acLDL with or without the MAPK p38 inhibition was done by confocal microscopy. Daily supplementation of the specific MAPK p38 inhibitor SB203580 (10 μM) was either started immediately before acLDL exposure (pre-acLDL) or at the very start of the pro-angiogenic culture until the end of acLDL exposure (start–finish). Representative pictures show reduced LD accumulation within monocytes after MAPK p38 inhibition (start–finish). The length of the scale bars is 10 μm . (B) Reduction of intracellular LD was found after SB203580 (dark gray bars) compared with controls (light gray, mean $\pm$ SD, $n = 3$).