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Amniotic liquid derived stem cells as reservoir of secreted angiogenic factors capable of stimulating neo-arteriogenesis in an ischemic model

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ABSTRACT

Most urgent health problems are related to a blood vessel formation failure. The use of stem cells from different sources or species for both *in vitro* and *in vivo* engineering of endothelium does not necessarily imply their direct commitment towards a vascular phenotype. In the present study, we used human amniotic fluid stem cells (AFSC) to evoke a strong angiogenic response in murine recipients, in terms of host guided-regeneration of new vessels, and we demonstrated that the AFSC secretome is responsible for the vascularising properties of these cells. We indentified in AFSC conditioned media (ACM) pro-angiogenic soluble factors, such as MCP-1, IL-8, SDF-1, VEGF. Our *in vitro* results suggest that ACM are cytoprotective, pro-differentiative and chemoattractive for endothelial cells. We also tested ACM on a pre-clinical model of hind-limb ischemic mouse, concluding that ACM contain mediators that promote the neo-arteriogenesis, as remodelling of pre-existing collateral arteries to conductance vessels, thus preventing the capillary loss and the tissue necrosis of distal muscles. In line with the current regenerative medicine trend, in the present study we assert the concept that stem cell-secreted mediators can guide the tissue repair by stimulating or recruiting host reparative cells.

1. Introduction

Blood vessel formation, either by *de novo* or from pre-existing ones, occurs in physiological processes such as embryonic development and wound healing or in pathological processes such as malignancies. Ischemic stroke, myocardial infarction, peripheral vascular disease, chronic wounds, macular degeneration are some of most urgent health problems related to blood supply deficiency. The creation of a functional vascular network for cellular perfusion within the implantation site [1] is one of the critical challenges also in "Tissue Engineering", since the survival of the implanted tissue constructs depends on sufficient oxygen and nutrient delivery to the cells [2].

The transplantation of different types of stem cells, potentially capable to differentiate into endothelial cells, was suggested as an alternative to treatments that are often non decisive or feasible such as anticoagulant drugs and surgery (angioplasty). Pluripotent human embryonic stem cells (hESCs) are a potentially unlimited source of endothelial cells [3], but their differentiation yield and purity need to be improved, their teratogenic risk and ethic concerns need to be well assessed and the functional outcome of hESC transplantation need to be enhanced in terms of cell engraftment and cell survival.

Endothelial-like cells were also obtained by differentiating human bone marrow-derived mesenchymal stem cells (MSC), human umbilical cord blood (UCB) -MSC and term amniotic membrane -MSC [4–7]; nevertheless, the maintenance of endothelial phenotype, once deprived of VEGF, and the full functionality of these derivative cells has not been proven yet.

Human amniotic liquid derived stem cells (AFSC) are a new and appealing source of stem cells. Moreover, they represent an attractive alternative to embryonic and adult stem cells because of their plasticity degree, safety, easily accessibility and minor ethical controversies [8]. Recently, AFSC were proposed as capable to differentiate into cells of the endothelial lineage. When cultured in growth medium supplemented with vascular endothelial growth factor, AFSCs expressed endothelial markers such as vWF and CD31 [9]. However, *in vitro* differentiation of AFSC toward a cellular type expressing vWF and CD31, cannot be considered a real commitment toward a vascular phenotype, at least before functional properties, such as tubule formation capability, uptake of acLDL or binding of ulex-lectin, are assayed. Furthermore, the obtaining of an *in vitro* differentiated stem cell population does not necessarily imply that these cells could resist and exert their functions once implanted *in vivo*.

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Here we studied AFSC not aiming at their direct differentiation into endothelial cells or their possible integration into the vascular endothelium after the implant, but considering their regenerative and angiogenic potential, i.e. their capacity to induce the formation of a new vascular network in the host. We used AFSC conditioned medium to evoke a strong angiogenic response in murine recipients, in terms of host guided-regeneration of new vessels, and we demonstrated that the AFSC secretome is responsible for their vascularising properties.

2. Materials and methods

2.1. Cell cultures and conditioned media

Human AFSC were received as confluent back up amniocentesis cultures from the Clinical- Cytogenetic Laboratory of Galliera Hospital (Genoa). Amniocentesis was performed between 15 and 17 weeks of gestation and only normal karyotyped-samples were transferred to our laboratory, after obtaining written informed consents from the women. Samples were pooled and c-Kit selected on a Mini-MACS apparatus (Miltenyi Biotec) as previously described [10]. AFSC were then expanded in αMEM medium (Gibco, Invitrogen) containing 15% ES-FBS, 1% glutamine and 1% penicillin/streptomycin (Gibco), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific), thereafter reported as "Chang MEM", at 37 °C within a 5% CO_2 atmosphere.

At a confluence of 70%, conditioned media of AFSC (ACM) were collected, after abundant washes in PBS and an incubation of 16 h in serum free αMEM (SFM). ACM were prepared and stored so as each millilitre of collected volumes derive from 500,000 AFSC. ACM with this concentration were used for all *in vitro* functional studies and *in vivo* applications.

HUVEC (Human Umbilical Vein Endothelial Cells) were purchased from IST- Cell Bank (http://www.iclc.it) and were cultured on 1% gelatine-coated flasks in M199 medium containing 10% FBS, 1% glutamine and 1% penicillin/streptomycin (Gibco), 10 ng/ml FGF-1, 10 ng/ml FGF-2, 10 ng/ml EGF (Peprotech), 1 ug/ml Hydrocortisone, 16 U/ml Heparin (Sigma), thereafter reported as complete endothelial medium (CEM).

2.2. Characterization of AFSC secretome

Conditioned media were obtained as described above from initially plated 150,000 AFSC/well (6-multiwell plate) and were assayed by a multiplex cytokine analysis at Luminex (X-Map Techology) with a customized panel of analites (Panomics), including VEGF, TGF β, MCP-1, IL-1α, IL-6, IL-8, SDF-1α, IFNγ, IP10. Obtained cytokine concentrations (pg/ml) were normalized for the DNA content (μg) of the relative secreting cell layers. DNAs were extracted using AllPrep DNA Mini Kit (Qiagen), following manufacturer's instruction, and quantified in μg by measuring the absorbance at 260 nm with a spectrophotometer.

2.3. In vitro functional studies

To study the capability of ACM to stimulate the differentiation of endothelial cells toward tubes, 20.000 HUVEC were plated on each well of a 24 multi-well plate, previously coated with 4 mg/ml Matrigel (BD). HUVEC were cultured for 16 h in presence of conditioned and unconditioned (serum free, SFM) media; CEM were used as positive control. Media were then removed and cells fixed with 3.7% PFA for 10 min and Giemsa-stained. Tube network formation was assessed by counting the number of branch points per view field on five random fields under a microscope (x 50 total magnification).

To study the presence of chemoattractants for endothelial cells in the ACM, a chemotaxis assay was performed on Boyden chambers using 12 μm-pore filter (Millipore). HUVEC were seeded at a density of 150.000 cells/well in the upper chamber in serum free M199. In the lower chamber the three different conditions (SFM, ACM and Umbilical Cord Fibroblast conditioned medium) were added. Chambers were incubated for 10 h at 37 °C, 5% CO_2. After removing non-migrating cells, the lower side of the filters was fixed in ethanol 96%, stained with 2% Toluidine Blue and the migrated cells counted on 5 random fields (×200 total magnification) per filter.

To study the effect of ACM on endothelial cell viability an MTT assay was performed. HUVEC were plated at the initial density of 30.000 cells/well (24-multiwell plate). The day after the three different media (SFM, ACM, CEM) were added and HUVEC cultured for 3 days. Cell viability was assayed at 0, 24, 48, 72 h by adding an MTT solution (Sigma) at final concentration of 0.25 mg/ml to each well. After an incubation of 2 h and 30 min at 37 °C, the formed formazan was dissolved in 1 ml Absolute EtOH and absorbances measured (OD_{570}-OD_{670}).

Finally, HUVEC were cultured in ACM and SFM for 16 h and tested for the expression of some surface molecules in a Cyan ADP Cytofluorimeter (Beckman Coulter). Monoclonal antibodies used for this study were: anti-CD31 (89D3, IgG2a, kindly provided by M. R. Zocchi, San Raffaele Scientific Institute), anti-CD146 (P1H12, IgG1 from BD PharMingen), anti-CD105 (IgG1) and anti-CD73 (IgG2b), purchased from the American Type Culture Collection (ATCC, Manassas, VA), followed by the addition of anti-isotype specific goat anti-mouse (GAM) antisera (Southern Biotechnology) conjugated with phycoerythrin (PE) (Invitrogen). Control samples were stained with isotype-matched irrelevant mAb (Becton Dickinson) followed by anti-isotype specific GAM-PE.

2.4. Animals

We used inbred male NOD-SCID mice aged 10–12 weeks from IST- animal facility. All animal experiments were approved by the regulatory board for animal welfare of IST animal facility and Italian Ministry of Health and are in line with European Union guidelines. We chose only male and immune-compromised mice to exclude the confounding effect of female cyclic estrogens on cardiovascular system, as well the effects of immune-recognition of human proteins, and subsequent inflammatory reactions, on vascular response.

2.5. In vivo assay for angiogenic activity of conditioned media

Angiogenic properties of AFSC were macroscopically observed on implanted AFSC-seeded biomaterials (polymers and gels) and on AFSC-treated wounds of skin. To assess if these properties could be attributed to soluble factors secreted by AFSC and in order to compare AFSC secretome to that of another stem cell source, a matrigel plug assay was performed. Conditioned media (1 ml derived from 500.000 cells for all the conditioned media tested) were concentrated at least 10-fold using Centricon YM-3 (Amicon). Concentrated medium was then added to a final volume of 600 ul liquid matrigel (12 mg/ml). Subcutaneous injections in mice were performed according to a published procedure [11]. Each mouse was injected with three matrigel plugs (in the back and in the two flanks with rotation of the plug position) containing: SFM, ACM and Bone Marrow Stromal Cell- Conditioned Medium (BMCM) or, in a second set of experiments, SFM, ACM and 500.000 AFSC. No heparin or other pro-angiogenic addictives were used. Five days after injection the matrigel sponges were recovered. A quantitative assessment of vascularization rate was done measuring the haemoglobin (Hb) content of matrigel plugs with Drabkin method (consumables from Sigma). Briefly, plugs were minced, dispersed in water to lyse erythocytes and centrifuged. Colour reaction of recovered supernatants was evaluated with a spectrophotometer at a wavelength of 540 nm. Haemoglobin values were normalized to 50 mg.

2.6. Pre-clinical animal model

To investigate ACM-mediated vascular regeneration, we chose a mouse model of hind-limb ischemia. Ischemia was created by unilateral ligation of the right femoral artery immediately distal to the origin of the deep femoral branch, following a surgical procedure previously published [12]. The treatment was started 10 days after the ligation. Mice were randomly divided into two groups (5 animals per group): PBS-treated and ACM-treated. Conditioned media from 500.000 AFSC were concentrated to the final volume of 80 μl, used for injections. Mice received two intramuscular weekly injections (topically applied to thigh muscles) for a total treatment-duration of two weeks (see the scheme in Fig. 4A). During this period recovery and functionality of the treated limb were monitored by timing the animal mobility across a 35 cm length tube. After sacrifice, mice were dissected to recover semimembranosus (SM) muscle from medial thigh muscles and gastrocnemius (GC) muscle from calf muscles (12). Arteriogenesis and distal angiogenesis plus muscular degeneration were determined by histomorphometry on SM and GC muscles respectively.

2.7. Histomorphometry

Briefly, muscles were fixed in buffered neutral formalin 10% (Sigma) over night, dehydrated in ethanol 70% (2 h), ethanol 80% (2 h), ethanol 96% (2 h), isopropanol (2 h), cleared in xilene and embedded in paraffin into an upright position.

Histomorphometry of collateral arteries was performed on at least five 7 μm thick-cross sections of SM muscle, previously stained with Mallory's trichrome (Bioptica). The arterioles density, the thickness of the mural wall and the total perfusion area, calculated as sum of lumen areas of endomysial arterioles per section, were measured using the Axiovision Rel 4.4 software and a Axiovert 200 microscope (Zeiss).

Capillary density (number of capillaries to number of muscular fibers) was determined on at least five 4 μm thick-cross sections of GC muscle. Immunohistochemistry was performed using a polyclonal anti-vWF (Dako) and a rabbit anti-murine CD31(Abcam) and staining with peroxidase method.

Degeneration and atrophy of muscular fibers were assessed on at least 3 H&E stained cross-sections of GC muscle. Axiovision Rel 4.4 software was used to measure fiber diameter, number of fibers per muscle fascicle (as number of counted events), and fascicle perimeter. Distribution curves of fiber diameters and a ratio of number of fibers per mm of fascicle perimeter were generated for each group of treatment.

2.8. Statistical analysis

Results are expressed as mean ± S.D. Statistical significance was assessed by use of unpaired t test for comparison between 2 independent treatments. We considered $P < 0.05$ to be significant.