ELSEVIER

A Novel Approach to the Identification of Genes Involved in Neo-Angiogenesis: Implications for Graft Re-Vascularization

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CELL transplantation is viewed as a potential alternative to vascularized allografts in selected diseases. The survival of grafted, nonvascularized tissues or cells is dependent on revascularization by the host's newly formed blood vessels.[1] As yet poorly defined angiogenetic/chemotactic factors originating from the grafted tissues may critically affect the revascularization process.[1,2,3] Our aim is to characterize the mechanisms that regulate endothelial cells proliferation and differentiation, which are at the basis of neo-angiogenesis. To investigate these processes, we use as a model the regulation of gene expression in contact inhibited endothelial cells versus actively proliferating cells.

MATERIALS AND METHODS

Cell Cultures

Human endothelial cells isolated from umbilical vein (EC) as previously described[4] were plated on 2% Gelatin-precoated tissue culture flasks and grown in long confluent (96 h) or subconfluent state. The proliferation rate of cells was measured by propidium iodide DNA staining followed by flow cytometry analysis, as described.[5] EC were grown in the presence of Medium 199 (GIBCO) containing 20% heat inactivated FBS, 1% I-glutamine, penicillin/streptomycin, 100 μg/mL heparin, 1% home-made RDGF (Retinal Derived Growth Factor), 1% Nutridoma (Boehringer). The medium was changed every 72 hours. The last change of medium was made 48 hours before the experiment.

RNA Preparation and Differential Display Analysis

Total RNA was isolated from confluent and subconfluent EC using RNazol (Biotecx). RT-PCR was performed using an oligo-dT primer. ^{32}P-labelled PCR amplification of the obtained cDNAs was carried out using random dodecamers as internal primers in a modified differential display PCR.[6] Amplification was carried out in the presence of 200 μmol/L dNTP, 4 μM primer, 1.5 mmol/L $MgCl_2$, with the following protocol: 94°C, 3 minutes; 94°C, 30 seconds; 42°C, 1 minute; 72°C, 1 minute (35 cycles); and 72°C, 5 minutes. The amplified products were displayed on denaturing polyacrylamide gels, differentially expressed bands were eluted, re-amplified with the same primers and cloned into pBluescript.[6,7] The cloned products were checked for differential expression by Dot-Blot hybridization using the original amplified cDNAs as probes. Differential expression was confirmed by performing Northern blot analysis on the original RNA, using the cloned product as specific probe. Products of interest were sequenced and compared to existing nucleic acid sequences in the Genebank data bases. A human EC cDNA library (Uni-ZAP XR vector, Stratagene) was screened for the identification and cloning of the full length coding sequence of the mRNAs of interest.

RESULTS AND DISCUSSION

A modified differential display PCR assay, using internal random primers, revealed a different pattern of cDNA expression between confluent (>90% GO/G1 arrested) and subconfluent (>50% S/G2/M) endothelial cells. We have cloned four different cDNA fragments: three appear to be more expressed in confluent growth arrested cells, and one in proliferating endothelial cells. All these four cDNA have been sequenced. One of these shows high homology with a intracellular protease inhibitor belonging to the MASPIN family, while the others have no homology with known genes. We are currently screening an endothelial cDNA library to obtain the full length cDNAs. Transfection of these full length cDNAs into quiescent or proliferating endothelial cells will allow us to determine their possible involvement in the regulation of endothelial cell proliferation and differentiation.

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0041-1315/97/$17.00
PII S0041-1345(96)00450-2