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cDNA Cloning of Human Allograft Inflammatory Factor-1: Tissue Distribution, Cytokine Induction, and mRNA Expression in Injured Rat Carotid Arteries

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We have previously identified a partial cDNA fragment obtained from rat carotid arteries subject to balloon angioplasty which is increased in response to vascular injury. We now report the isolation and characterization of a full length human clone which is 98% homologous at the amino acid level to the rat Allograft Inflammatory Factor-1 (AIF-1), a gene previously reported to be expressed in rat IFNγ-activated macrophages. This transcript is expressed in low levels in undamaged, and increases 1 day and 3 days, and declines 7 days post balloon angioplasty. This human AIF-1 homologue is inducible in serum and cytokine-stimulated human smooth muscle cells. Interestingly, human tissue distribution indicates a constitutive expression in lymphoid tissue which can be augmented by mitogens. In summary, human AIF represents a cytokine inducible, tissue-specific, and highly conserved transcript transiently expressed in response to vascular trauma.

The long term efficacy of percutaneous transluminal coronary angioplasty (PTCA) as a treatment for advanced multi-vessel coronary artery disease is significantly limited by the high incidence of vascular restenosis observed in as many as 40% of patients undergoing this procedure (1). The neointima formation associated with balloon angioplasty is a complex process which involves several different cell types which secrete many different cytokines and growth factors seminal to the local inflammatory response. These factors include, but are not limited to interleukin 1 (IL-1β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and a number of colony stimulating factors (CSFs) and interferons (IFNs) (2-4). The major cellular component of the atherosclerotic lesion is the vascular smooth muscle cell (VSMC), which, in response to vessel wall trauma migrates into the intimal layer and proliferates (5). The identification and characterization of proteins involved in modulation the VSMC response to injury and thus represents an important step in understanding the pathology of vascular restenosis.

We have previously identified a partial cDNA fragment obtained from mRNA Differential Display of rat carotid arteries subject to balloon angioplasty termed RC9 (6). We have also identified and characterized a previously unidentified full length homologue of RC9 transcript from rat testes termed BART-1 (7). Examination of the human tissue distribution of this transcript under low-stringency hybridization conditions drove us to obtain the full-length, human homologue from a peripheral blood lymphocyte cDNA library. Expression of this transcript, in balloon-damaged rat carotid arteries is similar to RC9 and BART-1, and the coding region is highly conserved. The amino acid sequence of this gene, however, is highly homologous the previously described rat Allograft Inflammatory Factor-1 (AIF-1) (8). In this work, we describe the human tissue distribution of human AIF-1, and its temporal expression pattern in balloon angioplasty-injured rat carotid arteries. We also demonstrate serum-and cytokine-inducible mRNA expression in human vascular smooth muscle cells, as well as mitogen-inducibility in human peripheral blood lymphocytes.

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MATERIALS AND METHODS

Rat left common carotid artery balloon angioplasty. Left common carotid artery balloon angioplasty was performed on 350g male Sprague-Dawley rats (Charles River Breeding Laboratory Inc., Wilmington, MA) under sodium pentobarbital anesthesia (65 mg/kg, i.p.; Steris Laboratories, Phoenix, AZ) as described previously (6). Briefly, the left external carotid artery was cleared of adherent tissue allowing the insertion of an 2-F Fogarty arterial embolectomy catheter (Model 12-060-2F; Baxter Healthcare, Santa Ana, CA). The catheter was guided a fixed distance down the common carotid artery to the aortic arch, inflated with a fixed volume of fluid and withdrawn back to the site of insertion a total of three times. Once completed, the catheter was removed and the wound closed (9 mm Autoclips; Clay Adams) and swabbed with Povadyne surgical scrub (7.5% Povidone-Iodine; Chaston, Dayville, CT). Animals were housed in Plexiglas cages under a 12 hour light/dark cycle with access to standard laboratory chow and drinking water *ad libitum* until required for tissue collection.

To isolate the carotid arteries, rats were exsanguinated *via* the vena cava under barbiturate anesthesia (100 mg/kg, i.p.). Left common carotid arteries were rapidly cleared of adherent tissue *in situ*, isolated and placed directly in guanidine thiocyanate (Promega Co., Madison, WI). These vessels were then immediately processed for RNA isolation. For subsequent Northern analysis, tissues were isolated from naïve animals (control) and from animals that had undergone angioplasty 1, 3 and 7 days prior, and RNA extracted as described in a following section. Northern analysis was also performed on sham vessels (data not shown). All surgical procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Deborah Research Institute and the American Association for Laboratory Animal Care.

Cells and culture. Human vascular smooth muscle cells (VSMC) were obtained as cryopreserved secondary culture from Clonetics Corporation (San Diego, CA) and subcultured in growth medium as described previously (9). The growth media was changed every other day until cells approached confluence. Cells from passages 5-9 were used in the described studies. Pre-confluent VSMCs were serum starved for 48 hours in Dubecco's minimum essential media, then exposed for 20 h. to 10% fetal calf serum, 10 ng/ml basic Fibroblast Growth Factor (bFGF), 100u/ml Interferon gamma (IFNγ) 20 ng/Ml Interleukin 1 beta (IL-1β), 20 ng/Ml Platelet Derived Growth Factor (PDGF), or 2 ng/mI Transforming Growth Factor beta (TGFβ) for 20 hours, at which times samples were processed for RNA isolation. Some samples remained untreated and used as controls. PDGF, bFGF, IFNγ, and TGFβ were purchased from GIBCO-BRL, (Bethesda, MD), and IL-1β purchased from Boehringer Mannheim (Indianapolis, IN). Human peripheral blood lymphocytes (PBL) were isolated by venipuncture from normal adult donors, isolated by Ficoll-Hypaque density-gradient centrifugation and cultured in DMEM/CM phytohemagglutinin A (PHA) (2.5 μg/ml) (purchased from Pharmacia) for the times indicated, and processed for RNA isolation.

cDNA library screening. A human PBL cDNA library (Clonetech, Inc.) was screened at moderate stringency (0.5$\times$ SSPE at 60°C) with the 424 bp cDNA fragment RC9. This probe was used to screen approximately 112,000 plaques, of which four were positive after three rounds of screening. Restriction digestion identified these as identical, and the preliminary identity of one clone as the RC9 homologue verified by dideoxy nucleotide sequencing using a sequence-specific primer. This clone had an insert of approximately 0.65 kb flanked by *EcoRI* sites as part of the vector polylinker.

DNA sequencing and sequence analysis. The cDNA clone obtained above was dideoxy nucleotide sequenced on both strands in its entirety (Sequenase, United States Biochemical Corporation) as previously described (7). DNA and protein sequences were analyzed using the Mac Vector software package (International Biotechnologies, Inc.). Searches for sequence similarity were performed using the GenBank Nucleic Acid Data base, and Prosite protein database through the Genetics Computer Group FASTA and BLAST programs.

RNA isolation and Northern blot analysis. For each time point studied, four or five left carotid arteries were pooled, or VSMC from culture isolated and total RNA obtained by standard techniques as described (6). Equal amounts of RNA were loaded and separated on a 1.3% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized (0.25M NaCl, 1% sodium dodecyl sulfate, 50% formamide, 2$\times$ Denhardt's solution, 25μg denatured salmon sperm DNA, 5% dextran sulfate 42°C overnight) with the indicated probe. All probes were [α^{32}P]-labeled by the random priming method (Boehringer Mannheim, Indianapolis, In.) (all isotopes were from Amersham Inc., Arlington Heights, IL.). Blots were washed under high stringency (0.2$\times$ sodium citrate, 0.1% sodium dodecyl sulfate, 65°C), and exposed to film for 6 - 48 hr. at $-$80°C. The same filter was stripped and subsequently hybridized with the various DNA probes. The beta actin probe was generated from PCR amplimers (Clonetech, Palo Alto, CA.). Relative intensities of hybridization signals were obtained by densiometric scanning (RFLP-Scan Software, Scanalytics, Inc.) of autoradiograms exposed within the linear range of the film (Kodak X-OMAT). Human multiple tissue Northern blots were purchased from Clonetech, Inc. (Palo Alto, CA), and hybridized and washed according to manufacturers instructions.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from treated and untreated cells as described above and 4μg was reverse transcribed using random hexamers as described previously (10). One-fifth of the cDNA was PCR amplified using the following primers: 5′ TATCATGTCCTTGAAACGAATGCTGGAGAA 3′, and 5′ TTTGTCTTCTGTTTTAGCATTCGGTCTCAG 3′ which define a 330 bp region of the human AIF cDNA. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) amplimers were purchased from Clonetech (Palo Alto,

CA)., and define an amplicon of 450 bp. One fifth of the reaction was run on a 2.5% agarose gel, ethidium-bromide stained, and photographed. PCR products were Southern transferred to hybridization membrane and hybridized with an end-labeled 45-mer oligonucleotide probe complementary for sequence internal to the PCR amplicons.

Genomic DNA species analysis. Genomic DNA Southern blots containing 4 μg of genomic DNA from nine different animal species digested was purchased from Clonetech, Inc. (Palo Alto, CA) and was hybridized at high stringency (5× sodium citrate, 10% Denhardt's solution, 100mg/ml sheared salmon sperm DNA, 1% sodium dodecyl sulfate, 65°C, overnight) with the full-length AIF-1 cDNA clone according to manufacturers instructions. Blots were washed under moderate (0.5× sodium citrate, 0.1% sodium dodecyl sulfate, 55°C) stringency and audioradiographed as described in the figure legend.

RESULTS

A previously described balloon angioplasty responsive transcript isolated from a rat testes cDNA library termed BART-1 (7) hybridized at low-stringency to mRNA isolated from several human tissues (unpublished data). The human tissue distribution of this transcript drove us to obtain the human homologue through screening of a peripheral blood lymphocyte cDNA library. Approximately 112,000 plaques from human peripheral blood lymphocyte cDNA library (Clonetech, Inc.) were screened at moderate stringency with a cDNA fragment corresponding to both the RC9 and BART-1 cDNA sequence. Three clones, each containing an approximately 700 bp insert were obtained and shown to be identical by restriction analysis. One was chosen and shown to be homologous to RC9 and BART-1 by dideoxy nucleotide sequencing. To fully identify this gene, the insert was subjected to dideoxynucleotide sequencing, and the nucleic acid and predicted protein sequence of this cDNA clone is depicted in Figure 1A. Following termination codons in all three reading frames, a 429 bp open reading frame was identified which follows the transcription initiation codon at position 86. A 112 bp 3′ untranslated region was also identified which contains a consensus polyadenylation signal AATAAA at position 618.

The 429 bp open reading frame encodes for a deduced 143 amino acid protein with a mass of approximately 16,318 kDa. This protein demonstrates an 83% amino acid sequence identity to the previously described rat allograft inflammatory factor-1, a gene expressed in activated rat macrophages (8). Human AIF-1 is four amino acids shorter than rat AIF-1, with the differences in amino acids residing primarily in the carboxy terminus of the protein. Amino acid motif analysis of the human AIF-1 protein reveals a consensus EF-hand loop domain that is a conserved feature of calcium-binding proteins (11). Other motifs include a potential Casein Kinase II phosphorylation consensus sequence (S)xx(D)x (12) located at amino acids 39-43, and a potential (PKC) site (S/T)x(R/K) at amino acids 69-71 (13). Despite the relatively high percentage of amino acids predicted to be serine residues, (10% of the amino acids) there are no predicted consensus phosphorylation sites for serine/threonine protein kinases including cAMP-dependent protein kinase, MAPK, or tyrosine kinases (12,14). The human AIF-1 protein does not possess any consensus immunoglobulin-like or cysteine rich regions (12,15), and there are no other signatures that suggest a potential function for this protein.

To determine if the mRNA expression of this clone in injured arteries was similar to that of RC9, total RNA from undamaged rat carotid arteries and from carotid arteries isolated at three time points following balloon angioplasty was performed with the insert of this clone as a hybridization probe (Figure 2). Scanning densiometric analysis of this blot normalized beta actin content demonstrates an 8-fold increase in expression of AIF-1 mRNA over basal levels 1 day post balloon angioplasty, 9-fold at 3 days, and 1.5-fold at 7 days post-injury. This expression pattern is consistent with that observed for the RC9 differential display fragment, with the exception of a basal level detected in naive vessels, and indicates that expression of this gene is induced in rat carotid arteries in response to balloon angioplasty.

We further investigated a mechanism for AIF-1 induction in vascular injury by stimulating human VSMC with a variety of growth factors, and examining AIF-1 expression by semi-

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                                                    GGACGGAGGGCACGAGAGAAGGAGA   25
    CGCTGCAGAAAGAGGCCTCCAGCTTGGTCTGTCTCCCACCTCTACCAGATCTGCTGAGCT   85
    ATGAGCCAAACCAGGGATTTACAGGGAGGAAAAGCTTTCGGACTGCTGAAGGCCCAGCAG  145
  1   M  S  Q  T  R  D  L  Q  G  G  K  A  F  G  L  L  K  A  Q  Q
    GAAGAGAGGCTGGATGAGATCAACAAGCAATTCCTACACGATCCCAAATATAGCAGTGAT  205
 21   E  E  R  L  D  E  I  N  K  Q  F  L  H  D  P  K  Y  S  S  D
    GAGGATCTGCCCTCCAAACTGGAAGGCTTCAAAGAGAAATACATGGAGTTTGACCTTAAT  265
 41   E  D  L  P  S  K  L  E  G  F  K  E  K  Y  M  E  F  D  L  N
    GGAAATGGCGATATTGATATCATGTCCTTGAAACGAATGCTGGAGAAACTTGGAGTCCCC  325
 61   G  N  G  D  I  D  I  M  S  L  K  R  M  L  E  K  L  G  V  P
    AAGACTCACCTAGAGCTAAAGAAATTAATTGGAGAGGTGTCCAGTGGCTCCGGGGAGACG  385
 81   K  T  H  L  E  L  K  K  L  I  G  E  V  S  S  G  S  G  E  T
    TTCAGCTACCCTGACTTTCTCAGGATGATGCTGGGCAAGAGATCTGCCATCCTAAAAATG  445
101   F  S  Y  P  D  F  L  R  M  M  L  G  K  R  S  A  I  L  K  M
    ATCCTGATGTATGAGGAAAAAGCGAGAGAAAGGAAAACCAACACGCCCCCCAGCCAAGAA  505
121   I  L  M  Y  E  E  K  A  R  E  R  K  T  N  T  P  P  S  Q  E
    AGCCCTATCTGAGATGCCTGATTTGAGGGAAAAGGGATGATGGGATTGAAGGGGTTCTAA  565
144   S  P  I
    TACCCAGATATGGAAACAGAAGACAAAATCGTAAGCCAGAGTCAACAAATTAAATAAATT  625
    TACCCCAAAAAAAA                                                639
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B

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human RC9   MSQTRDLQGGKAFGLLKAQQEERLDEINKQFLHDPKYSSDEDLPSKLEGF  50
consensus   MSQ++DLQGGKAFGLLKAQQEERLD INK FL DPKYSSDEDL SKLE F
rat AIF     MSQSKDLQGGKAFGLLKAQQEERLDGINKHFLDDPKYSSDEDLQSKLEAF  50

human RC9   KEKYMEFDLNGNGDIDIMSLKRMLEKLGVPKTHLELKKLIGEVSSGSGET 100
consensus   K KYMEF DLNGNGDIDIMS LKRMLEKL GVPKTHLELKKLI EVSSGS ET
rat AIF     KTKYMEFDLNGNGDIDIMSLKRMLEKLGVPKTHLELKKLIREVSSGSEET 100

human RC9   FSYPDFLRMMLGKRSAILKMILMYEEKARERKTNTPPSQESPI        143
consensus   FSY DFLRMMLGKRSAIL+MILMYEEK +E +  T P  +  I
rat AIF     FSYSDFLRMMLGKRSAILRMILMYEEKNKEHQKPTGPPAKKAISELP  147
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FIG. 1. (A) Nucleic acid and deduced amino acid sequence of human AIF-1 (GenBank Accession Number U49392). Following termination codons in all three reading frames, a 429 bp open reading frame follows the ATG at position 86. Potential phosphorylation consensus sequences for Casein Kinase II at amino acids 39-43, PKC at amino acids 69-71 are in bold type. A consensus polyadenylation signal AATAAA at position 618 is also underlined. (B) Amino acid homology of human RC9 to rat AIF-1 (GenBank Accession Number U17919). Consensus residues are in bold type, and conservative amino acid substitutions are marked by a +. The boxed area includes the EF-hand-like motif, and the shaded area represents the conserved calcium-binding loop.

quantitative reverse transcription polymerase chain reaction (RT-PCR). In these experiments, VSMC were grown to just under confluency, serum deprived to quiescence, and exposed to 10% fetal calf serum (FCS) basic Fibroblast Growth Factor (bFGF), Interferon gamma (IFNγ), Interleukin-1 beta (IL-1β), Platelet Derived Growth Factor (PDGF), or Transforming Growth Factor beta (TGF-β). As compared to untreated cells, human AIF-1 is upregulated in both FCS and cytokine treated VSMC (Figure 3A). The identity of this amplicon as human AIF-1 was confirmed by Southern analysis of the same gel hybridized with a probe specific for sequence internal to the AIF-1 amplimers (Figure 3B). Interestingly, less of a product is observed in the bFGF-treated cells as compared to other factors examined. These results indicate that in human VSMC, AIF-1 is a cytokine-responsive transcript.

A previous report indicates that rat AIF-1 mRNA is tissue specific, being expressed only in rat spleen, macrophages and testes. Figure 4 demonstrates that in contrast to the rat isoform, human AIF-1 is expressed in a variety of human tissues, with the highest expression in cells of lymphoid origin, in particular, spleen, peripheral blood lymphocyte, and thymus. A lesser degree of expression is detected in liver, lung, and placenta. Also in contrast to rat AIF-1, no

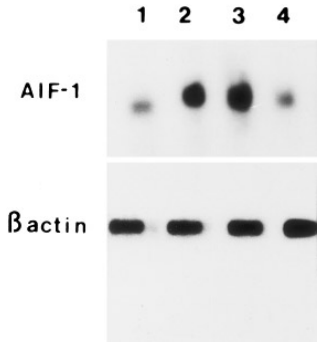

FIG. 2. Northern analysis of RNA from rat left common carotid arteries subject to balloon angioplasty prior to, 1, 3, and 7 days following balloon angioplasty (lanes 1, 2, 3, and 4, respectively) probed with a human AIF-1 DNA probe. Total RNA (10 μg) from rat carotid arteries was separated on a 1.3% agarose/formaldehyde gel, transferred to nitrocellulose, hybridized, and washed as described in the methods. A beta actin probe was used as a loading control.

expression is detected in human testes. The relatively high degree of constitutive expression of AIF-1 in human lymphoid tissue suggests a function for the AIF-1 protein in cells of this lineage.

The inducibility of AIF-1 in VSMC taken together with its high degree of expression in human lymphoid cells drove us to investigate expression of this transcript in activated human PBL as well. Figure 5 is a semiquantitative RT-PCR analysis of the human AIF-1 transcript in phytohemagglutinin A (PHA)-stimulated human PBL, and demonstrates that 24 hr treatment of these cells with PHA increases AIF-1 accumulation, and this mRNA level is maintained at 72 hr post-stimulation. These results indicate that the constitutive levels of AIF-1 mRNA expression in human PBL can be augmented by the lymphocyte mitogen PHA.

The observation that AIF-1 mRNA is expressed in activated VSMC and PBL led us to investigate if expression of this gene was linked to cellular proliferation. Therefore, we examined expression of AIF-1 mRNA in spontaneously proliferating transformed cancer cell lines. Figure 6 shows that human AIF-1 mRNA is expressed in only two of eight human cancer cell lines examined; lymphoblastic leukemia MOLT-4 and promyeleocytic leukemia HL-60 (lanes 5 and 8, respectively). The expression of AIF-1 in MOLT-4 cells is also in contrast to that reported for the rat transcript (8). The lack of expression in all cell types examined suggest that expression of this gene is not strictly linked to cellular proliferation.

Because as yet no function has been ascribed to the AIF-1 protein, the evolutionary conservation of this sequence may offer some information as to its importance. Southern blot analysis was carried out using *EcoRI*-digested genomic DNA from a variety of animal species including human, monkey, rat, mouse, dog, cow, rabbit, chicken, and yeast (*Saccharomyces cerevisiae*).

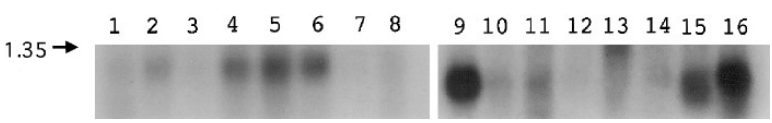

FIG. 3. Northern analysis of human tissue distribution of human AIF-1 mRNA expression. Two μg poly A^+ mRNAs from pancreas (1), kidney (2), skeletal muscle (3), liver (4), lung (5), placenta (6), brain (7), heart (8), peripheral blood lymphocyte (9), colon (10), small intestine (11), ovary (12), testes (13), prostrate (14), thymus (15), and spleen (16) were hybridized with each respective probe as described in methods. The respective size of the transcript is 0.7kb, and size standards in kb are indicated by numbers on the right of the figure. The blot was purchased from Clonetech, Inc. La Jolla, CA.

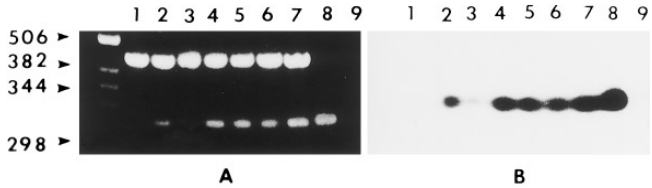

FIG. 4. Expression of human AIF-1 cDNA in activated human vascular smooth muscle cells (VSMC). (A) Semi-quantitative RT-PCR was performed on 4μg total RNA isolated from 1-serum starved, and VSMC treated for 24 hours with 2- 10% FCS, 3- bFGF, 4- IFNγ, 5- IL1-β, 6- PDGF, and 7-, TGFβ using human AIF-1 and G3PDH amplimers defining amplicons of 330 and 450 bp, respectively, as described in materials and methods. One fifth of the reaction was run on a 2.5% agarose gel, ethidium-bromide stained, and photographed. Lane 8 is a positive control from the plasmid, and lane 9 is a no Reverse Transcriptase template control. Size markers in bp are indicated to the left of the figure. (B) Southern analysis of the same gel hybridized with a probe specific for sequence internal to the AIF-1 amplimers. The film was exposed for 5 minutes.

The AIF-1 coding region cDNA probe hybridized to a number of restriction fragments from various species under low moderate washing conditions (0.5× SSC, 0.1% SDS, 55°C), the most prominent being three bands of approximately 4.4, 2.3, and 2.2 kb in human DNA (Figure 7). A number of strong bands of less than 2 kb in length are visible in mouse DNA, and other bands of lesser intensity were detectable in rat, dog, and cow. Interestingly, no clear banding pattern is observed in lane 2, monkey. A number of minor bands are detectable in chicken and yeast, suggesting that the AIF-1 protein coding region is well conserved. Additional studies will be necessary to rule out multiple copies of this gene.

DISCUSSION

Neointima formation subsequent to balloon angioplasty is the result of a dynamic process actively involving several different cell types and occurring in several phases. The initial response is primarily inflammatory in nature involving T-lymphocytes and macrophages which secrete many different cytokines and growth factors seminal to the local inflammatory response. The second phase involves the principle cellular component of the restenotic lesion, the vascular smooth muscle cell (VSMC), which in response to these factors migrates into the intima, secretes soluble growth and chemotactic factors, extracellular matrix, and proliferates. The third phase may last several weeks post-injury and is characteristic of chronic fibroproliferative lesion formation. Although the cellular response to balloon angioplasty is fairly well characterized, the lack of effective anti-restenotic pharmacological adjuncts to prevent the neointima formation associated with PTCA is indicative of our poor understanding of the precise molecular mechanisms underlying this disease process.

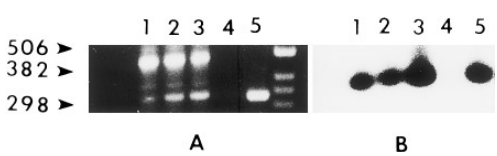

FIG. 5. Expression of human AIF-1 cDNA in activated human peripheral blood lymphocytes (PBL). (A) Semi-quantitative RT-PCR was performed on 4μg total RNA isolated from 1-unstimulated, and 2- 24 hr, 3 -72 hour phytohemagglutinin-stimulated PBL using human AIF-1 and G3PDH amplimers defining amplicons of 330 and 450 bp, respectively, as described in materials and methods. One fifth of the reaction was run on a 2.5% agarose gel, ethidium-bromide stained, and photographed. Lane 4 is a no Reverse Transcriptase template control, and lane 5 is a positive control from the plasmid. Size markers in bp are indicated to the left of the figure. (B) Southern analysis of the same gel hybridized with a probe specific for sequence internal to the AIF-1 amplimers. The film was exposed for 5 minutes.

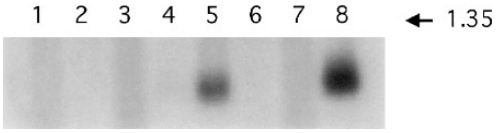

FIG. 6. Expression of human AIF-1 in eight human cancer cell lines. Two μg poly A^+ mRNA from: 1; melanoma G361, 2; lung carcinoma A549, 3: colorectal adenocarcinoma SW480, 4; Burkitt's lymphoma raji, 5; lymphoblastic leukemia MOLT-4, 6; chronic myelogenous leukemia D-562, 7; HeLa cell S3, and 8; promyeleocitic leukemia HL-60. Size standards in kb are indicated by numbers on the right of the figure. The blot was purchased from Clonetech, Inc. La Jolla, CA.

We have previously described several genes whose expression is modulated in response to balloon angioplasty of rat carotid arteries (6,7). Since the expression of the gene termed RC9 appeared to be species conserved, we screened a human PBL library and obtained the full length human homologue (Figure 1A). Sequence identity searches in the GenBank/EMBL database indicate a high degree of homology to the rat AIF-1 gene, (Figure 1B), as well as several short, unidentified sequences in the GenBank EST database.

Amino acid analysis of this protein demonstrates a 28 amino acid region corresponding to an EF-hand helix-loop motif. The EF-hand calcium-binding motif is present in many calcium binding proteins, where it is assumed to play a role in modulation of Ca^{2+} activation, Ca^{2+} buffering, and structural stabilization (11,16,17). Myosin light chains are members of the EF-hand superfamily and it has been shown that at least in these proteins, the EF-hand motif may bind divalent cations to potentially regulate contraction. This observation may partially explain the expression of this gene in injured arteries and activated VSMC (Figures 2 and 3, respectively).

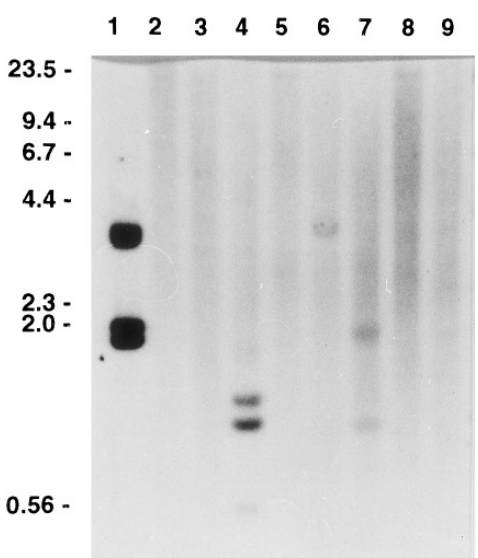

FIG. 7. Evolutionary conservation of human AIF-1 cDNA. Southern blot analysis was carried out using *EcoRI*-digested genomic DNA (4 μg) from a variety of animal species including 1- human, 2- monkey, 3- rat, 4- mouse, 5- dog, 6- cow, 7- rabbit, 8- chicken, and 9- yeast (*Saccharomyces cerevisiae*). Size standards in kb are indicated by numbers on the right of the figure. The probe was hybridized to the filter at 65°C according to manufacturers directions, and washed at low stringency stringency (2× SSC, 0.5% SDS, 45°C). The blot was purchased from Clonetech, Inc. La Jolla, CA.,

The rat AIF-1 gene was observed to be expressed exclusively in the monocyte infiltrate of allografted rat hearts (8). In this system, low level expression was not observed until 7 days post implant, and peaking at 28 days post procedure. These levels declined to approximately half-maximal at 72 days post-implant. This expression appeared to coincide with the degree of activated macrophages in this tissue. In balloon-damaged rat carotid arteries, we observed increased expression 1 day post angioplasty, peaking at 3 days, and declining by one week (Figure 2). Although a leukocyte infiltrate is a significant cell type in restenotic lesions, because activated VSMC are the primary cell type in restenotic lesions, and since many of the cytokines found in the restenotic lesion are VSMC growth factors, we hypothesized that expression of this transcript was derived from a more abundant cell type such as VSMC.

In VSMC, every cytokine with the exception of bFGF tested induced AIF-1 mRNA (Figure 4). However, not all of these cytokines are proliferative for VSMC, particularly IFNγ (1,4,18). Further, the increase above constitutive levels in mitogen-stimulated PBLs may be due to autocrine expression of lymphocyte growth factors such as IL-2 in such stimulated cells (14). Therefore, we examined spontaneously proliferating human cancer cells to delineate between cytokine-induced and proliferation associated AIF-1 expression. Although by definition these are growing cells, AIF-1 message was not observed in all lines tested (Figure 6). Although this observation may reflect a cell-type specificity of AIF-1 expression, taken together with the observation that VSMC treated with non-proliferative cytokines still express AIF-1, it may be concluded that AIF-1 may represent a cytokine-responsive rather than proliferation-responsive transcript.

While some proteins of the EF-hand family, such as calmodulin, are ubiquitously expressed, most display a tissue-specific expression pattern, with the concomitant heterogeneity in the promoter regions of these genes (11). The human AIF-1 gene displays such a tissue specificity, with the greatest abundance of mRNA constitutivley expressed in cells of lymphoid lineage (Figure 4). This tissue-restricted expression, in conjunction with cytokine-inducibility in VSMC, suggests complex cis and trans factors governing AIF-1 mRNA expression. Further, this also infers dual functions for the AIF-1 protein; a constitutive role in maintenance of the leukocyte phenotype, and a second role in cytokine-activated VSMC. In light of the results obtained from the genomic zoo blot (Figure 7), this function is important enough for the sequence to be conserved through many species.

With the exception of the EF-hand motif itself, very little amino acid homology is displayed among the hundreds of proteins that belong to the EF-hand family, suggesting a secondary function in addition to calcium binding. However, aside from putative Ca^{2+} binding, the function(s) for the vast majority of these proteins is unknown. The function of the AIF-1 protein in cytokine-stimulated human VSMC and lymphocytes, as well as the mechanisms of regulation of its expression remains to be elucidated.

ACKNOWLEDGMENT

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