Eotaxin and Capping Protein in Experimental Vasculopathy

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Ischemia-induced tissue activation may contribute to the pathogenesis of graft vasculopathy, but the mediators implicated have only partially been characterized. To gain further insight into the molecular mechanisms involved, syngeneic rat aortic transplants with cold-storage-induced vasculopathy were studied for differentially expressed mRNA transcripts. Vessel segments were exposed to either 1 or 18 hours of cold ischemia, followed by transplantation into syngeneic recipients. After 3 days or 4 weeks, the grafts were removed and total mRNA was isolated and used for differential display to identify modulation of transcript expression related to prolonged storage. Using 15 sets of random primers, 17 polymerase chain reaction products were up-regulated and 2 were down-regulated in grafts exposed to 18 hours of ischemia. Sequencing of these amplicons showed that 6 had a high degree of homology to known sequences whereas 13 had no homology to any of the genes in the database. Two of the differentially displayed amplicons (capping protein and eotaxin) were cloned, re-amplified, and used as probes for Northern blot analysis to confirm their differential expression. Immunohistochemistry using monoclonal antibodies against capping protein- α and eotaxin confirmed that both proteins are expressed in the media of normal aortas and that there was an increased expression in vessels exposed to prolonged ischemia albeit that the increase at the protein level seemed less compared with changes in transcript expression. Northern blots with RNA from aortic allografts exposed to prolonged ischemic storage also showed increased levels of capping protein and eotaxin mRNA whereas there was a decrease in the relative amount of these transcripts in vessels exposed to balloon denudation, suggesting that the increase after prolonged ischemic exposure is not the result of a nonspecific response to injury. Based on the biological characteristics of capping protein and eotaxin it is conceivable that they play a

pathogenetic role in ischemia-induced vessel wall remodeling. It remains to be established whether these genes or their products serve as target molecules for therapeutic interventions to prevent or treat cold-storage-induced graft vasculopathy. (Am J Pathol 1998, 153:81-90)

Chronic rejection and graft vasculopathy are the principal cause of organ transplant failure after the first post-transplant year. Immunological and nonimmunological mechanisms have been implicated in both the initiation and the progression of the disease, but the exact contribution of each of these factors remains poorly understood. Most forms of chronic graft failure seem to converge along a common complex pathway involving lipid mediators, cytokines, chemokines, growth factors, and enzymes involved in the regulation of cell cycle progression and extracellular matrix metabolism.¹

With improvements in immunosuppressive medication it is expected that the impact of ischemia-reperfusion injury on long-term graft outcome becomes more important. Ischemia-reperfusion injury is one of the nonimmune events that enhances chronic rejection,² induces vasculopathy in syngeneic vessel grafts,³ and adversely affects the long-term outcome of organ transplants.⁴ The pathophysiology of ischemia-reperfusion injury is complex and involves local hemodynamic factors, depletion of high-energy phosphates, and generation of reactive oxygen species, enzymes, cytokines, and vasoactive mediators (reviewed in Ref. 5). In transplanted organs, it also enhances the immunogenicity of the grafted tissue and aggravates allogeneic immune injury.

The molecular mediators of graft vasculopathy in general and ischemia-induced graft vasculopathy in particular are incompletely defined. As modulation of steady-state mRNA expression represents a key molecular determinant in many biological processes, we used the differential mRNA display technique to gain insight into which genes are differentially expressed in grafts with ischemia-induced vasculopathy. Using this approach, we reported previously on quantitative differences in ex-

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pression of various genes in rat aortic allografts with immune-induced vasculopathy.⁶

Materials and Methods

Transplants

Male PVG (RT1^c) and DA (RT1^a) rats were purchased from Möllegaard (Skensved, Denmark) and housed in the vivarium at either the University Hospital in Uppsala or at the Transplantation Laboratory at the University of Helsinki. The animals had free access to tap water and standard rat chow, and all procedures were performed in accordance with the institutional guidelines, the European Convention order 85/90, and order 86/609 of the European Economic Community. The study was approved by the Animal Care Review Committees at the respective institutions.

Aortic transplants were done as described.³ Briefly, the abdominal aorta was removed from the donor under general anesthesia after careful ligation of the vessels that arose from it, followed by flushing with a perfusion solution and storage at 4°C. Recipients were anesthetized with a chloral hydrate/pentobarbital mixture, and the abdominal aorta was clamped; using a 9-0 continuous nylon suture, the aortic graft was anastomosed end-to-end to the recipient aorta below the renal arteries. Post-operative pain medication consisted of buprenorphine. Grafts were removed at 3 days or 4 weeks and used for histopathology and RNA isolation. For some experiments, additional tissue specimens were obtained for immunohistochemistry at 2 hours or 8 weeks.

Arterial Injury Model

Endothelial denudation and vessel wall injury were produced in the left common carotid artery, as described by others.⁷ Briefly, rats were anesthetized by intraperitoneal administration of sodium pentobarbital and the region of the left carotid bifurcation and distal common carotid artery were exposed. A Fogarty 2F balloon embolectomy catheter was introduced through the external carotid artery, advanced into the thoracic aorta, followed by filling of the balloon with 0.008 to 0.012 ml of water to distend the vessel and to cause resistance to the withdrawal of the catheter; the balloon catheter was subsequently slowly withdrawn with a twisting motion. After three repetitions of this procedure, the endothelium was completely removed and the superficial layers of the media had sustained mild to moderate injury. After removal of the catheter, the incisions were closed. Tissue samples were obtained 0, 3, 7, or 14 days after denudation for the isolation of RNA.

RNA Isolation and Differential Display

Total cellular RNA was isolated using the guanidinium thiocyanate-caesium chloride method.⁸ Samples used for differential mRNA display were treated with DNase I to remove genomic DNA; 20 µg of total RNA was incubated

for 30 minutes with 10 U of DNase I (MessageClean kit, GenHunter, Boston, MA). After extraction with phenol/CHCl₃ (3:1), the supernatant was ethanol precipitated in the presence of 0.3 mol/L sodium acetate (NaOAc), followed by suspension of the RNA in diethyl pyrocarbonate (DEPC)-water. A 0.5-µg amount of total RNA was used for reverse transcription in the presence of one of three polyT primers (H-T₁₁A, H-T₁₁C or H-T₁₁G; GenHunter) and Moloney murine leukemia virus reverse transcriptase as follows: 65°C for 5 minutes and 37°C for 10 minutes followed by addition of the reverse transcriptase and subsequent incubation at 37°C for 50 minutes. The reaction was stopped by heating to 95°C for 5 minutes. Control reactions were done in the absence of reverse transcriptase. One-tenth of the reverse transcription product was used as a template for polymerase chain reaction (PCR) amplification with 15 sets of arbitrary 10-mer 5' primers and the same set of 3' H-T₁₁ primers (RNA-image) in the presence of α-³⁵S-labeled dATP (1200 Ci/mmol; Amersham, Arlington Heights, IL) and *Taq* DNA polymerase (AmpliTaq, Perkin Elmer, Norwalk, CT). The reaction was performed at 94°C for 30 seconds, 40°C for 2 minutes, and 72°C for 30 seconds for 40 cycles. The amplified DNA products were separated on a denaturing 6% polyacrylamide gel. All reactions were repeated twice and analyzed in neighboring lanes to limit false positive signals. Differentially up-regulated genes were defined as those DNA bands that were consistently present in tissue samples exposed to either 1 hour or 18 hours of ischemia but not in both.

Recovery and Re-Amplification of DNA Probes

Polyacrylamide gels were dried onto Whatman 3-mm paper without fixation. The autoradiogram and the dried gel were oriented with radioactive ink, and the DNA bands of interest were located and isolated by cutting through the film. The gel slice, along with the paper, was incubated in 100 µl of dH₂O for 10 minutes; to facilitate the diffusion of DNA out of the gel, the sample was boiled for 15 minutes. The DNA was recovered by ethanol precipitation in the presence of 0.3 mol/L NaOAc with 5 µl of 10 mg/ml glycogen as a carrier, followed by re-dissolving it in 10 µl of dH₂O. A 4-µl aliquot of the DNA probe was re-amplified in a 40-µl reaction volume using the same primer set and PCR conditions as used in the mRNA display, except that the dNTP concentration was 20 µl/L and no isotope was added. The PCR samples (30 µl) were run on a 1.5% agarose gel stained with ethidium bromide. Re-amplified DNA bands were purified by cutting them out and extracting the gel slice using the QIAEX kit from QIAGEN (Hilden, Germany).

Northern Blot Analysis

Twenty micrograms of total RNA was resolved on a formamide 1% agarose gel and transferred onto nylon membranes (Amersham Hybond N). Probes were prepared by metabolic labeling of the re-amplified DNA fragment with [³²P]dCTP (3000 Ci/mmol; Amersham)

using the random primer DNA-labeling kit from GIBCO (Gaithersburg, MD) according to the manufacturer's instructions. Prehybridization and hybridization was done in 50% formamide, 5X sodium chloride, sodium hydroxyphosphate, ethylenediamine tetra-acetate (SSPE), 0.5% sodium dodecyl sulfate (SDS), 5X Denhardt's, and 100 μ g/ml denatured salmon sperm DNA (GIBCO). After overnight hybridization, the filter was washed with 6X SSPE, 0.1% SDS at room temperature two times for 15 minutes each, followed by 1X SSPE, 0.1% SDS at 37°C two times for 15 minutes each. The autoradiogram was developed by exposure to hyperfilm (Reflection, DuPont, Wilmington, DE), followed by quantitation using a video densitometer (Bioquant system IV; R&M Biometric, Nashville, TN). The loading of RNA onto the gel was standardized by comparing the signal intensity with that obtained with the probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Sequencing

PCR amplicons that were differentially expressed were sequenced using the Cyclist Exo(-) Pfu DNA sequencing kit from Stratagene (La Jolla, CA). Approximately 50 ng of QIAEX (QIAGEN) purified DNA was used as a template in combination with 3 ng of one of the arbitrary primers used for the differential display. After denaturing the DNA at 95°C for 5 minutes, the sequencing reaction was cycled for 30 rounds through the following temperatures using a DNA thermal cycler (Perkin Elmer 480): 95°C for 30 seconds, 45°C for 30 seconds, and 72°C for 50 seconds. The reaction was ended by adding 5 μ l of stop buffer, and 8 μ l of the sample was heated at 80°C for 2 minutes before loading onto a 6% sequencing gel. Autoradiograms were developed by exposing the dried gel to hyperfilm (Amersham) for 2 days. Sequences were determined and their homology was compared with known sequences by searching databases using the BLAST program.

Cloning

Two amplicons of interest were re-amplified using the same set of primers as the original ones under the following conditions: 94°C for 1 minute, 45°C for 1 minute, and 72°C for 30 cycles followed by 72°C for an extra 7 minutes. One microliter of the PCR-amplified products was mixed with 2 μ l of pCR 2.1 vector (TA cloning kit, Invitrogen Corp., Carlsbad, CA) in the presence of ligation buffer and T4 DNA ligase. The ligation reaction was kept overnight at 14°C. Two microliters of the ligation mixture was added to INV α F' One Shot competent cells in the presence of β -mercaptoethanol. After 30 minutes on ice, the reaction mixture was heat-shocked for 30 seconds in a 42°C water bath followed by 2 minutes on ice; 250 μ l of SOC medium was added to the mixture and shaken at 37°C for 1 hour. Either 50 or 200 μ l of the transformation mixture was spread onto LB agar plates containing 50 μ g/ml ampicillin and X-Gal, respectively; the plates were incubated at 37°C in an incubator for 18

hours, followed by 2 to 3 hours at 4°C. White colonies were selected and grown up in 3 ml of LB-ampicillin overnight in a shaker at 37°C. Plasmid DNA was prepared from the bacterial lysates using the QIAprep Spin Plasmid kit (QIAGEN). The cloned insert and its orientation were determined using the T7 sequencing kit from Pharmacia (Uppsala, Sweden). Briefly, 2 μ l of plasmid DNA was denatured in NaOH and precipitated in 100% ice-cold ethanol. Template DNA was mixed with universal primers in annealing buffer and incubated under the following conditions: 65°C for 5 minutes, 37°C for 10 minutes, and 25°C for 5 minutes. The labeling and termination reactions were performed according to the manufacturer's instructions. Two microliters of the sequencing mixture was loaded on a 6% sequencing gel, followed by autoradiography of the dried gel.

Immunohistochemical Staining

Acetone-fixed, 5- μ m-thick frozen sections were incubated overnight at 4°C with primary antibodies dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin. A rat-absorbed biotinylated anti-mouse IgG antibody (1:80; Vector Laboratories, Burlingame, CA) served as the secondary antibody, and an avidin-biotin complex (Vector) method was used for its detection. The peroxidase reaction was carried out with 3-amino-9-ethylcarbazole as substrate, and counterstaining was performed with Mayer's hematoxylin.

mAb5B12.3 is a mouse IgG antibody obtained by immunization of BALB/c mice with GST-capping protein α 1 subunit from chicken. The antibody reacts with capping protein α 1 and α 2 subunits from various species, including chicken, mouse, and human. This antibody was a gift from Dr. John Cooper, Department of Cell Biology and Physiology, Washington University Medical School, St. Louis, MO. LS59 10C11 is a mouse IgG2a monoclonal antibody against eotaxin and was a gift from LeukoSite Inc., Cambridge, MA.⁹

ED-1 antibodies recognize rat monocytes and macrophages; the antibodies were purchased from Serotec Laboratories, Oxford, UK, as in previous experiments.^{3,6}

The slides were read by one of us without prior knowledge of the experimental groups, the differential display, or Northern blot results. The extent of staining was scored on a scale ranging from 0 to 3, where 0 indicates absence of antigen positive cells and 3 represents heavy tissue infiltration by antigen-expressing cells. Scoring was done without prior specific knowledge of the experimental groups. The Mann-Whitney *U* test was used for statistical comparisons.

Results

Differential Display and Sequencing

Previous studies have shown that cold storage of rat aortic grafts for 4 hours or longer results in intimal thickening within 4 weeks after syngeneic transplantation.³ For the present experiments, total RNA was isolated from

Figure 1. Examples of differentially expressed genes in syngeneic aortic grafts transplanted after 1 or 18 hours of cold storage and removed on day 3 or week 4 after transplantation. **Arrows** indicate amplicons that were up-regulated in grafts exposed to 18 hours of ischemia but not in grafts exposed to 1 hour of ischemia; the up-regulated amplicon in **A** shows sequence homology with rat eotaxin; the up-regulated amplicon in **B** shows sequence homology with capping protein.

rat aortic grafts exposed to 1 or 18 hours of cold storage followed by transplantation into a syngeneic recipient from which it was removed 3 days or 4 weeks later to examine differences in steady-state mRNA expression. Using 15 sets of primers, 19 differentially expressed cDNA clones or amplicons were identified; 17 were expressed in grafts exposed to 18 hours of cold storage but not in grafts exposed to 1 hour of cold storage whereas 2 were found in grafts stored for 1 hour but not in grafts stored for 18 hours. Figure 1 shows two representative examples of differentially expressed amplicons in grafts exposed to 1 or 18 hours of cold storage.

All 19 amplicons that were differentially expressed were isolated and sequenced by direct asymmetric PCR, followed by homology searches. The sequences of six amplicons were highly homologous to known sequences: *Rattus norvegicus* eotaxin (total length of the reference cDNA was 1001 bp; 62 of 71 bp between 723 and 793 (87%), 54 of 54 bp between 665 and 718 (100%), and 22 of 24 bp between 634 and 657 (91%) were homologous), *Mus musculus* capping protein $\alpha 1$ subunit (total length of the reference cDNA was 2893 bp; 124 of 128 bp between 2736 and 2863 (96%) and 27 of 30 bp between 2864 and

2893 (90%) were homologous), *Rattus norvegicus* pituitary suppressin (total length of the reference cDNA was 1882 bp; 205 of 210 bp between 1402 and 1611 (97%) were homologous), mouse mRNA for a dexamethasone-induced product (total length of the reference cDNA was 573 bp; 100 of 105 bp between 468 and 572, 64 of 64 bp between 340 and 403 (100%), and 30 of 31 bp between bp 440 and 470 (96%) were homologous), *Mus musculus* clone DE-5 mRNA fragment (61/61), and a novel gene expressed in an apoptotic T-cell hybridoma (53/54). Thirteen amplicons, including the two that were expressed in grafts exposed to 1 hour but not 18 hours of cold storage had no sequence homology with any known genes in the database.

Northern Blot Analysis with PCR-Amplified Fragments

As the differential display technique has a high false positive rate,⁶ we sought to confirm the differential expression of eotaxin and capping protein using Northern blots. The amplicons of interest were labeled with ³²P and

Figure 2. Northern blots of [³²P]dCTP-labeled eotaxin (**A**), [³²P]dCTP-labeled capping protein (**B**), and [³²P]dCTP-labeled GAPDH (**C**) probes with RNA prepared from normal aortas or syngeneic aortic grafts exposed to 1 or 18 hours of cold storage and removed 3 days after transplantation. There was a 1.7- and a 9-fold increase in the eotaxin/GAPDH ratio between normal aortas and syngeneic aortic grafts exposed to 1 hour or 18 hours of storage, respectively (**A**). There was a 1.1- and 3.4-fold increase in the capping protein/GAPDH ratio between normal aortas and syngeneic aortic grafts exposed to 1 or 18 hours of cold storage, respectively (**B**).

used for Northern analysis with RNA isolated from normal PVG aortas and syngeneic grafts exposed to 1 hour or 18 hours of cold storage. Figure 2 shows the Northern blots with RNA isolated from normal aortas and aortic grafts 3 days after implantation and confirms that the expression of both gene products was enhanced with prolonged ischemic exposure. There was a 1.7- and 9-fold increase in the eotaxin/GAPDH ratio between normal aortas and syngeneic aortic grafts exposed to 1 hour or 18 hours of storage, respectively; the increase between 1 and 18 hours of storage was 5.3-fold (Figure 2A). There was a 1.1- and 3.4-fold increase in the capping protein/GAPDH ratio between normal aortas and syngeneic aortic grafts exposed to 1 or 18 hours of cold storage, respectively (Figure 2B). Similar increases in eotaxin and capping protein mRNA were observed in DA aortic grafts exposed to either 1 or 18 hours of ischemia before transplantation into allogeneic PVG recipients and removed at 3 days after implantation; the eotaxin/GAPDH mRNA ratio increased 1.6-fold and the capping protein/GAPDH ratio increased 1.4-fold (data not shown).

To study whether the increase in eotaxin and capping protein mRNA was specific for cold storage, we studied carotid arteries that had been exposed to balloon damage. Figure 3 shows a representative example of Northern blots with RNA prepared from denuded vessels that shows a decrease in eotaxin/GAPDH mRNA in samples obtained 0, 3, 7, or 14 days after denudation; mean eotaxin/GAPDH mRNA ratio on these days were 2.34, 1.09, 1.25, and 1.94, respectively, reflecting a twofold decrease compared with day 0 samples. These results were confirmed in another experiment (data not shown). The capping protein/GAPDH mRNA ratio also decreased in carotid arteries removed on day 3 after denudation (2.6 versus 5.2 on day 0) but had returned to day 0 values on day 7 (5.1 on day 7 and 4.2 on day 14) (Figure 3). These results were confirmed in another experiment (data not shown).

Cloning

To ensure that the amplicons used were cDNAs from one gene product, the amplicons were cloned into the pCR 2.1 vector and resequenced; two clones of each gene of interest were sequenced. Figure 4 shows homology for 222 of 222 bp between bp 578 and 799 (100%) and 18 of 21 bp between bp 783 and 803 (85%) of the rat eotaxin gene. Figure 5 shows homology for 124 of 128 bp between 2736 and 2863 (96%) and 27 of 30 between 2864 and 2893 (90%) of the *Mus musculus* capping protein α 1 subunit. No other mammalian cDNAs were detected.

Immunohistochemistry

Immunoperoxidase staining was done on normal and ischemic aortic grafts to study the tissue localization of eotaxin and capping protein and to assess whether changes in respective mRNA expression corresponds with changes at the protein level, as assessed by immunohistochemistry. Between four and six specimens per

Figure 3. Northern blots using [32 P]dCTP-labeled eotaxin (A), [32 P]dCTP-labeled capping protein (B), and [32 P]dCTP-labeled GAPDH (C) probes with RNA prepared from carotid arteries exposed to balloon injury and removed on days 0, 3, 7, or 14 after injury. The mean eotaxin/GAPDH ratios were 2.3, 1.1, 1.2, and 1.94 on days 0, 3, 7, and 14, respectively; the mean capping protein/GAPDH ratios were 5.2, 2.6, 5.1, and 4.2 on days 0, 3, 7, and 14, respectively.

group at each time point were examined. Staining of aortic graft sections with the anti-eotaxin monoclonal antibody LS5910C11 showed that eotaxin is weakly expressed in the media of normal rat aorta. In grafts exposed to 18 hours of cold storage, a few eotaxin-positive cells were found in the intima and adventitia as early as 2 hours after transplantation; a similar expression pattern was observed in grafts that remained *in situ* for a longer period of time (Figure 6). However, at the protein level, the changes in eotaxin protein expression were not significant (Figure 7).

Normal aortas expressed capping protein in the media but not in the intima or adventitia (Figure 8). Both 1 and 18 hours of cold storage resulted in an increase in capping protein expression in both the intima and adventitia, but the increase was significant only in grafts exposed to 18 hours of cold storage (Figures 8 and 9). Co-localization studies using serial sections and an antibody against

R_EOTAXI T7_6_2	GTGTGTGAT TTCTTGATA TGCAACATCT TAAAAGG-AG -AGAGC-AT	546 22
R_EOTAXI T7_6_2	GGA-A---GG AGATGTTGGA TT---A-TT GAGGATAA--GA-----A	578 72
R_EOTAXI T7_6_2	AGGAATGAGT TAGGGGTAAC TGAGGATGAT AGGAGGACTA AATACTATCA	628 122
R_EOTAXI T7_6_2	AAGCACCTTA TACACATATG AAAATGTCAT AACATGACCC ATTGTTTTTC	678 172
R_EOTAXI T7_6_2	AAAATTAATA TACACTGATT AAAATGCTAA TATTTAAGGA CATGTTACAT	728 222
R_EOTAXI T7_6_2	TTAAGAAATT GGAATTTTAA AGCATATTTT AATGGATCTT GGTTCCTTTT	778 272
R_EOTAXI T7_6_2	GTGTTTTCG TTGTTTGCTT GTTTGAAATA GGGTCTCACT GTATCACCTT	828 298

Figure 4. Sequence homology of the cloned eotaxin amplicon showing a high degree of homology with *Rattus norvegicus* eotaxin cDNA.

smooth muscle cell α -actin (Figure 8D) showed that most of the capping-protein- α -positive cells are smooth-muscle-actin-positive cells although macrophages may also express the protein as evidenced by the presence of capping-protein- α -positive cells in large-sized mononuclear cells in the adventitia surrounding suture material (Figure 10). Co-localization studies using serial sections and the ED-1 antibody showed only sporadically ED-1-positive cells in the intima (Figure 8E).

Discussion

Graft arteriopathy, the hallmark of chronic rejection, results from vascular remodeling in response to transplantation-related vessel wall damage.¹⁰ Whereas immune factors play an important role, the possible impact of ischemia-reperfusion injury has only recently been investigated.¹¹ Clinical studies have shown a detrimental effect of prolonged cold storage,¹² ischemic damage, and delayed graft function^{13,14} on the long-term survival of

kidney transplants. Syngeneic rat aortic grafts exposed to prolonged cold storage develop vasculopathy that resembles the intimal hyperplasia after balloon injury and to some extent allogeneic grafts with vasculopathy except that in allogeneic vasculopathy there is media necrosis and infiltration of the intima by macrophages, T lymphocytes, and smooth muscle cells compared with smooth muscle cells and macrophages in ischemic syngeneic grafts.³ Differences in the composition of the graft-infiltrating cells under different conditions suggest differences in the response-to-injury pathways after different types of injury.

Comparison of differential mRNA expression between normal vessels and vessels exposed to various degrees of cold storage has the potential to provide information about the mediators involved in ischemia-reperfusion injury. Although several methods exist to study differential gene expression from one tissue to another, the differential mRNA display method is attractive because it requires only small amounts of RNA and is more sensitive

capping Complement of A3	CATTACATTT GTTTACACTT CTATGACCAG GCGT-TAGCG GA-AGAT--C	2646 22
capping Complement of A3	ATTTT-TTAA -AAA-A-CGA --AGTA-ENG TCTTCCTAIC TGTCCCGAG	2689 72
capping Complement of A3	TACAGGTAAG AACAG-AAAG GCGT-T-ENG CTTCACTTTT CTTTCTTTT	2735 121
capping Complement of A3	TTGTTTCAGTA ATACCTCAAG GCAGGAGTTT GTTTGCAAT GAAAGCTGTG	2785 171
capping Complement of A3	TAAGCATTTT TGTATTATTC AAATAAAATA TATTTGTATT ATTGTGCTAT	2835 221
capping Complement of A3	CATACTATCC ATCCATGCCA CACTGT-TTC TGTACCAGGT AGTCCAATAG	2885 268
capping Complement of A3	AAATAACCT GTTTTGTCT AAAAAAAAAA AGCTTAAAA	2893 307

Figure 5. Sequence homology of the cloned capping protein amplicon showing a high degree of homology with *Mus musculus* capping protein α -1 subunit.

Figure 6. Immunoperoxidase staining for eotaxin of a syngeneic aortic graft exposed to 18 hours of cold storage and 2 hours of residence in a syngeneic host. There is eotaxin expression in the medial smooth muscle cells.

than subtractive hybridization for the detection of rare transcripts.¹⁵ Using this approach, 19 differentially expressed amplicons were identified, 17 in syngeneic grafts exposed to 18 hours of cold storage but not in grafts exposed to 1 hour of cold storage, and 2 in grafts exposed to 1 hour of storage but not in grafts exposed to 18 hours of cold storage. Figure 1 illustrates two representative examples of amplicons differentially expressed in grafts exposed to 18 hours of cold storage. Sequencing of all differentially expressed amplicons showed that 13 had no homology to any of the genes in the data base and 6 had a nucleotide sequence that was highly homologous to known sequences although two amplicons were very short and likely not informative. The amplicons that were derived from the eotaxin and capping protein genes were further studied.

As an amplicon could consist of one or more cDNAs of the same molecular weight, the amplicons of interest were cloned and resequenced, which confirmed the initial finding that the amplicons were derived from mRNA of eotaxin and capping protein (Figures 4 and 5). Moreover,

as the differential display technique has a high false positive rate,⁶ their differential expression was confirmed by Northern blots (Figure 2).

Eotaxin is an eosinophil-specific chemoattractant of the Cys-Cys family of chemokines^{9,16} with a high degree of amino acid sequence homology with monocyte chemoattractant proteins (MCPs). Eosinophils express receptors for eotaxin, which also recognize MCP-3 and RANTES, as shown in binding and cross-sensitization experiments, but eotaxin binds with higher affinity and does not appear to interact with other known chemokine receptors.^{9,16,17} The human eotaxin receptor CCR3 has recently been demonstrated on a population of Th2-like cells generated *in vivo* or *in vitro*.¹⁸ In rodents and humans, eotaxin is found in conditions characterized by eosinophil accumulation, such as allergic inflammation.^{9,16,17} Eotaxin secretion is stimulated in cultured endothelial and epithelial cells by interferon and interleukin-4¹⁶ and in peripheral blood eosinophils by interleukin-3.¹⁹ Eosinophils are believed to be involved in tissue damage,^{20,21} and intragraft eosinophilia has been reported during acute and chronic kidney and liver graft rejection.²²⁻²⁷ Moreover, experimental studies in a mouse cardiac allograft model have suggested that acute rejection episodes characterized by a Th2-like cytokine pattern is mediated predominantly by eosinophils.²⁸

Northern blots with mRNA prepared from syngeneic aortic grafts exposed to 1 or 18 hours of cold storage showed a more than fivefold increase in eotaxin mRNA with prolonged cold exposure. A similar but less dramatic increase was found in allogeneic aortic grafts exposed to prolonged storage. Immunohistochemistry showed eotaxin expression in the medial smooth muscle cells of normal aortas. With cold storage, its staining intensity increased in the medial smooth muscle cells and to a lesser extent in the adventitia and intima, but the increases were not significant. There are several possibilities to explain this discrepancy between increased transcript levels and protein product, including translational and post-translational modifications.²⁹ Thus, it is possible that increased mRNA levels do not result in increased expression of the protein. However, it is also possible that the use of a low-affinity antibody with cross-species reactivity precludes an accurate assessment of changes in protein expression or that only very few cells, not detected by immunohistochemistry, are responsible for the increased mRNA levels.

To investigate whether the up-regulation of eotaxin mRNA was specific for cold storage, Northern blots were done on carotid vessels exposed to denudation. In all of these tissues we found decreased mRNA expression, suggesting that ischemia-associated increased eotaxin mRNA levels are part of a pathway activated by ischemia rather than part of a response to injury reaction. As we found no eotaxin expression in endothelial cells by immunohistochemistry, it is unlikely that the decrease in eotaxin message after denudation results from loss of endothelial cells. Instead, the increase in eotaxin expression in the ischemia model seems to result from increased production by medial smooth muscle cells.

Figure 7. Semiquantitative evaluation of eotaxin expression in the intima, media, and adventitia of syngeneic aortic grafts exposed to either 1 or 18 hours of cold storage and studied 2 hours, 3 days, or 4 weeks after transplantation. The changes in eotaxin expression assessed by immunoperoxidase are not significant.

Figure 8. Immunoperoxidase staining for capping protein α -1 subunit expression in a native aorta (A) and in a syngeneic aortic grafts after 1 hour (B) or 18 hours (C) of cold storage and α -smooth muscle actin (D) and monocyte (ED-1) staining (E) in syngeneic grafts exposed to 18 hours of cold storage. All grafts were removed 4 weeks after transplantation. A shows the constitutive expression of capping protein in the media of a normal aorta. B shows a few capping-protein-positive cells in the intima and media in grafts exposed to 1 hour of cold ischemia. C shows diffuse and pronounced expression of capping protein α -1 subunit in the neointima after 18 hours of ischemia. Staining of a serial section with anti-smooth muscle actin antibodies (D) or the anti-monocyte antibody ED-1 (E) shows that capping protein α -1 subunit expression is mostly in smooth muscle cells whereas there are only very few ED-1-positive cells in the intima. The arrow shows internal elastic lamina. Immunoperoxidase staining counterstained with Mayer's hematoxylin; magnification, $\times 400$.

Capping protein is a ubiquitous heterodimeric actin-binding molecule (reviewed in Ref. 30) that *in vitro* nucleates actin polymerization and caps the barbed end of filaments, preventing addition and loss of monomers.³¹ Its co-localization with actin filaments in various tissues and cell types³²⁻³⁴ suggests a similar function *in vivo*. Inhibition of capping protein's ability to bind actin alters the actin organization in muscle cells undergoing myofibrillogenesis.³⁵ Finally, cell lines with increased levels of capping protein exhibit limited polymerization and an increased rate of cell movement in response to chemoattractants.³⁶ Northern blots of aortas exposed to 18 hours of cold storage showed a threefold increase in capping protein mRNA compared with aortic grafts exposed to 1 hour of cold storage. Prolonged storage of allogeneic grafts also resulted in a modest increase in capping protein mRNA, comparable to the increase found for eotaxin mRNA. Immunohistochemistry showed weak expression of the capping protein in normal aortas in the medial smooth muscle cells but not in the intima or ad-

ventitia (Figure 8) and 18 hours of cold storage induced expression of capping protein in the intima and adventitia (Figures 8 and 9). Although macrophages may express capping protein (Figure 10), there were only sporadic macrophages present in the intima (Figure 8E), and the induced expression was predominantly in cells with the phenotypic appearance of smooth muscle cells (Figure 8, C and D). We speculate that the increased expression of smooth muscle capping protein in smooth muscle cells in grafts with vasculopathy is associated with migration of vascular smooth muscle cells into the intima and adventitia,³⁷ whereas its expression in macrophages³⁸ may be related to regulation of actin filament length and concentration required for macrophage locomotion, phagocytosis, and degranulation. Increased capping protein mRNA expression is not a nonspecific response to injury as we found decreased mRNA levels in nontransplanted arteries that had been exposed to balloon injury.

We have previously reported up-regulation of immunoglobulin J chain, ferritin heavy chain, and *ras* p21-like

Figure 9. Semiquantitative evaluation of capping protein α -1 subunit expression in the intima, media, and adventitia of syngeneic aortic grafts exposed to either 1 or 18 hours of cold storage and studied 4 weeks after transplantation. The 0 time denotes nontransplanted normal aortas. The increase in capping-protein-positive cells in the intima and adventitia is significant in the group exposed to 18 hours of cold ischemia.

small GTP-binding protein transcripts in allogeneic aortic allografts with graft vasculopathy following transplantation after standard ischemic storage.⁶ In ongoing experiments we are studying the expression of these transcripts in syngeneic vessel grafts with ischemia-induced intimal proliferation, and preliminary data show up-regulated expression of *ras* p21-like small GTP-binding proteins mRNA in these grafts. The ratio of *ras* p21-like small GTP-binding proteins over GAPDH mRNA in syngeneic grafts exposed to 1 or 18 hours of ischemia and studied at 1 month was 2.8- and 3.2-fold higher than in normal vessels (data not shown). These data are consistent with the hypothesis that *ras* p21-like small GTP-binding proteins play a role in smooth muscle cell proliferation, irrespective of the initiating insult. *In vitro* studies have established the critical functions of *ras* p21-like small GTP-binding proteins in cell growth and differentiation,³⁹ and recent *in vivo* studies have shown that local delivery of transdominant negative mutants of *ras* give 50% inhibition of intimal thickening after carotid artery denudation.⁴⁰

In this study we demonstrate increased expression of eotaxin, capping protein, and *ras* p21-like small GTP-

Figure 10. Accumulation of capping protein α -1 subunit-positive cells around surgical material in the adventitia of a syngeneic graft 8 weeks after transplantation, suggesting that some macrophages may also express capping protein α -1 subunit. Magnification, $\times 400$.

binding protein mRNA in syngeneic aortic grafts with ischemia induced vasculopathy. The increased expression of eotaxin and capping protein was associated with prolonged cold storage whereas the enhanced *ras* p21-like small GTP-binding protein mRNA expression appears related to intimal smooth muscle cell proliferation. However, the importance of these findings with respect to therapeutic interventions in vessel wall remodeling remains to be established.

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