Vascular Biology, Atherosclerosis and Endothelium Biology

Identification of Differential Protein Expression Associated with Development of Unstable Human Carotid Plaques

Mark Slevin,* Abdul Baset Elsbali,*
Marta Miguel Turu,† Jerzy Krupinski,†‡§
Lina Badimon,§ and John Gaffney*

From the Department of Biological Sciences,* Manchester Metropolitan University, Manchester, United Kingdom; the Department of Neurology,† Stroke Unit, University Hospital of Bellvitge, Barcelona, Spain; Instituto de Investigacion Biomédica de Bellvitge,‡ Barcelona, Spain; and Cardiovascular Research Centre,§ Consejo Superior de Investigaciones Científicas, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain

Rupture-prone unstable arterial plaques develop concomitantly with the appearance of intraplaque hemorrhage and tissue ulceration, in association with deregulation of smooth muscle cell mitogenesis and leakage of newly formed blood vessels. Using microarray technology, we have identified novel protein deregulation associated with unstable carotid plaque regions. Overexpression of proapoptotic proteins caspase-9 and TRAF4 was seen in endothelial cells and smooth muscle cells from unstable hemorrhagic and ulcerated plaque regions. Topoisomerase-II- α (TOPO-II- α), which is associated with DNA repair mechanisms, was also overexpressed by these cells. Cell signaling molecules c-src, G-protein-coupled receptor kinase-interacting protein (GIT1), and c-jun N-terminal kinase (JNK) were up-regulated in endothelial cells from the same areas, whereas an increase in expression of junctional adhesion molecule-1 (JAM-1) in blood vessels and infiltrating macrophages from inflammatory regions might form part of a leukocyte rolling response, increasing the plaque volume. Grb2-like adaptor protein (Gads), responsible for differentiation of monocytes into macrophages, was expressed by macrophages from unstable plaques, suggesting a potential mechanism through which increased scavenging could occur in rupture-prone areas. We conclude that modulation of novel cell signaling intermediates, such as those described here, could be useful in the therapy of angiogenesis

and apoptosis, designed to reduce unstable plaque formation. (Am J Pathol 2006, 168:1004–1021; DOI: 10.2353/ajpath.2006.050471)

Carotid artery atherosclerosis is the primary mediator of ischemic stroke, which is a leading cause of death and disability in the Western world. The development of symptoms follows conversion of stable plaques to unstable ones, concomitant with the appearance of intraplaque hemorrhage, fibrous cap thinning, and infiltration with macrophages and T cells, as well as surface ulceration and rupture and thrombosis. Specifically, unstable plaques may be identified by Echo-Doppler ultrasonography and histological American Heart Association criteria of recent ulceration and hemorrhage.¹ The pathobiological mechanisms responsible for this conversion include increased inflammation and angiogenesis as well as changes in the rate of and resistance of cells to apoptosis.^{1,2} The molecular mechanisms responsible for induction of these changes have not been fully described.

Elevated concentrations of proinflammatory markers including intracellular adhesion molecule-1,³ matrix metalloproteinase-9,⁴ interleukin-18,⁵ and tumor necrosis factor (TNF)- α ⁶ have been implicated in the progression of asymptomatic to symptomatic plaques. A reduction in expression of platelet-activating factor receptor suggests a reduction in the capacity for defense associated with loss of the macrophage inflammatory phenotype.⁷ Overexpression of growth factors and cytokines produced as a consequence of the inflammatory process induces signal transduction activity, which impacts on the critical pathways of angiogenesis and apoptosis within developing plaque tissue.

Angiogenesis is a recognized feature of the atherogenic process, with intimal neovascularization arising most frequently from the dense network of vessels in the

Accepted for publication November 10, 2005.

Address reprint requests to Mark Slevin, Department of Biological Sciences, Oxford Rd., Manchester, M1 5GD, UK. E-mail: m.a.slevin@mmu.ac.uk.

adventitia, adjacent to a plaque, rather than from the main artery lumen.² New blood vessels may have an active role in plaque metabolic activity and actively promote its growth beyond the critical limits of diffusion from the artery lumen.⁸ Later in the progression of disease, the inherent weakness of newly forming blood vessels could result in development of intraplaque hemorrhage and instability.⁹ A strong correlation between areas of increased vascularity and intraplaque hemorrhage has been demonstrated by histological staining with anti-CD34 in symptomatic patients after endarterectomy.¹⁰ The irregular nature of blood vessel formation has been likened to tumor angiogenesis, and hence the factors responsible for their growth may be different from those seen during normal wound healing.¹¹ Plaque-associated endothelial cells (ECs) become activated, associate with monocytes and macrophages, and show increased expression of adhesion molecules. Thus, they may also be at least partly responsible for induction of the inflammation associated with neurological symptoms.¹²

Apoptosis of the cellular components within susceptible atherosclerotic lesions can increase the likelihood of plaque rupture by increase in the lipid core volume, thinning the fibrous cap, reduction in concentration of interstitial collagen fibers [due to smooth muscle cell (SMC) apoptosis], and production of leaky microvessels (EC apoptosis), resulting in hemorrhage.¹³ Both vascular SMCs and ECs found in unstable lesions overexpress proapoptotic proteins such as Fas ligand and Bax with a propensity for down-regulation of anti-apoptotic counterparts such as Bcl-2.^{14,15} Animal models have been used to identify some deregulated intermediates. For example, plaques induced in cholesterol-fed rabbits demonstrated increased staining of both c-jun N-terminal kinase (JNK) and phosphorylated p53 in macrophages undergoing apoptosis in lesional cap and basal regions.¹⁶ Global gene apoptosis-specific microarray studies comparing nonatherosclerotic and atherosclerotic carotid arteries have also identified novel mediators including death-associated protein (DAP) kinase in foam cells of SMC origin.¹⁷

As mentioned above, the signaling mechanisms responsible for cellular changes resulting in plaque instability have not been fully determined. Previous studies have used global gene microarray analysis to measure changes in mRNA expression in plaque samples; however, evidence suggests that a significant number of gene products are not translated, and the correlation between mRNA and protein concentration is often poor.^{17–19} More recently, Western array analysis was used to compare protein expression in normal mammary artery and human carotid artery specimens.²⁰ The authors demonstrated down-regulation of a novel proapoptotic protein, apoptosis-linked gene-2 (ALG-2), in plaque samples and postulated that it could provide a survival mechanism and protect against plaque rupture.

In the present study, we have used protein microarrays to compare protein expression in carotid endarterectomy samples histologically defined as stable and unstable. Relevant deregulated proteins were confirmed by Western blotting, including comparison with a series of normal

carotid artery specimens obtained at transplant. We identified overexpression of several novel proteins that could impact both angiogenesis/cell proliferation (junctional adhesion molecule-1 or JAM-1, G protein-coupled receptor kinase-interacting protein or GIT1, and c-src) and apoptosis (topoisomerase-II- α or TOPO-II- α , caspase-9, TNF receptor-activating factor-4 or TRAF4, Grb2-like adaptor protein or GADS, and JNK), in promoting plaque instability and thrombosis.

Materials and Methods

Materials

Glass protein microarrays containing 512 antibody sets (in duplicate) were obtained from BD Biosciences (Clontech, Buckinghamshire, UK). Cy3/Cy5 NHS-esters used for protein labeling and disposable PD-10 desalting columns were from Amersham Biosciences, UK. The following antibodies used for Western blotting and immunohistochemistry were obtained from Autogen Bioclear (Wiltshire, UK) and were rabbit polyclonal unless otherwise stated: anti-c-src, anti-JAM-1 (mouse monoclonal), anti-GIT1, anti-TOPO-II- α , anti-JNK, anti-caspase-9, anti-TNF- α , anti-GADS, anti-TRAF4, EC marker anti-CD105 (R&D Systems, Abingdon, UK), SMC marker anti-SMC-actin (Sigma Chemicals, Dorset, UK), and macrophage marker anti-CD64. Bio-Rad protein detection reagent was from Bio-Rad (Hercules, CA). Vectastain ABC kit was from Vector Laboratories, UK. All other materials and chemicals were from Sigma Chemicals.

Carotid Endarterectomy Specimens

Human carotid endarterectomy specimens ($n = 17$) were obtained from patients with a significant degree of carotid stenosis ($>70\%$), as demonstrated by duplex ultrasonography. Extensive neurological examination was used to determine stable/asymptomatic/unstable/symptomatic subgroups. The lesion was considered symptomatic when associated with transitory or established neurological deficits. Computed tomography scans were considered positive when cerebral infarcts were present in the area of the ipsilateral territory of cerebral arteries. All patients had Echo-Doppler on admission (before surgery) and 6 months later. Clinical and biochemical details are listed in Tables 1 and 2. The specimens were opened longitudinally and divided into two pieces, one fixed in 10% formaldehyde and the other frozen at -80°C for use in protein arrays and Western blotting. Nonatherosclerotic carotid artery specimens ($n = 7$) were obtained from age-matched individuals and obtained during transplant bypass surgery. All patients gave consent for the use of their plaque and blood samples for research analysis. Tissue acquisition and subsequent use were approved by the local ethical committee.

Table 1. Characteristics of Study Patients Used in Microarray Studies and Western Blotting

Patient no.	Age, year	Sex, M/F	Stable (n = 5)	Unstable (n = 12)	Plaque ulceration	Intraplaque hemorrhage	Fibrotic	Symptomatic
22	78	M	Stable		Yes	Yes	No	Yes
46	76	F	Stable		Yes	Yes	No	No
26	67	M	Stable		No	No	Yes	Yes
33	67	M	Stable		No	No	Yes	Yes
34	58	F	Stable		No	No	Yes	Yes
23	68	M		Unstable	Yes	Yes	No	No
4	63	M		Unstable	No	No	Yes	Yes
6	70	M		Unstable	Yes	No	No	Yes
10	65	M		Unstable	Yes	No	No	Yes
28	79	M		Unstable	No	No	Yes	No
20	65	M		Unstable	Yes	No	No	No
45	55	M		Unstable	Yes	Yes	No	Yes
7	75	F		Unstable	No	No	Yes	No
21	71	M		Unstable	Yes	Yes	No	Yes
42	73	M		Unstable	No	No	Yes	No
48	68	M		Unstable	Yes	Yes	No	Yes
50	66	M		Unstable	Yes	Yes	No	No
Additional plaques examined by immunohistochemistry								
3	73	M	Stable	Unstable	Yes	No	Yes	Yes
29	71	M			Yes	Yes	No	No
0	70	M		Unstable	Yes	Yes	No	Yes
52	77	M		Unstable	Yes	Yes	No	Yes
54	52	M		Unstable	No	Yes	No	Yes
58	71	M		Unstable	Yes	Yes	No	No
5	57	M		Unstable	Yes	Yes	No	Yes
78	67	F		Unstable	Yes	Yes	No	No
113	77	M		Unstable	Yes	Yes	No	No
121	76	M		Unstable	Yes	Yes	No	Yes

Bold type indicates stable plaques and italicized type indicates unstable plaques whose protein was pooled in the microarray study.

Table 2. Clinical Details of Patients Used in This Study

Patient no.	Echo-Doppler	Cholesterol	Fibrinogen	CRP	Diabetes	Leukocytes ($\times 10^3$)
22	Hyperecho	6.5	5.5	6.7	No	8.9
46	Hyperecho	3.9	4.5	3.1	Yes	9.7
26	Hyperecho	4.0	4.0	13.6	Yes	9.0
33	Hyperecho	4.1	3.0	2.1	No	8.0
34	Hyperecho	3.9	5.0	61.7	No	10.5
23	Hyperecho	6.5	6.5	1.7	No	4.7
4	Hyperecho	5.4	5.7	2.4	Yes	16.2
6	Hyperecho	4.7	4.1	13.9	Yes	6.1
10	Hypoecho	6.0	3.1	4.9	No	6.6
28	Hypoecho	4.3	4.4	0.9	No	6.5
20	Hypoecho	5.2	3.4	2.2	Yes	6.1
45	Hypoecho	5.3	4.8	1.5	No	9.7
7	Hyperecho	5.9	5.5	86.5	No	3.2
21	Hypoecho	3.9	5.8	0.9	Yes	6.1
42	Hypoecho	6.2	4.1	2.3	No	7.5
48	Hypoecho	4.8	3.5	34.9	No	7.3
50	Hypoecho	4.9	4.1	31.6	No	7.0
Additional plaques examined by immunohistochemistry						
3	Hyperecho	4.6	3.5	0.5	No	6.3
29	Hyperecho	4.7	3.3	0.5	No	5.1
40	Hyperecho	4.5	2.2	0.9	Yes	6.3
52	Hypoecho	6.3	3.1	8.0	Yes	5.0
54	Hyperecho	5.4	6.1	0.2	No	5.3
58	Hypoecho	5.1	3.3	0.3	No	5.5
65	Hypoecho	5.3	3.4	4.3	No	9.6
78	Hypoecho	5.1	3.6	1.0	Yes	4.8
113	Hypoecho	2.8	3.2	2.5	No	5.2
121	Hypoecho	5.1	6.5	40.8	No	7.4

Bold type indicates stable plaques and italicized type indicates unstable plaques whose protein was pooled in the microarray study.

Table 3. Confirmatory Western Blotting Results Showing Relative Expression of Deregulated Proteins in Carotid Plaque Mixtures Compared with Normal Arteries

Protein	Function	Fold change compared to normal artery	
		Stable	Unstable
Caspase-9	Initiator caspase involved in mediating mitochondrion-dependent apoptosis ²⁷		2.5
Cleaved Gads	Grb2-like adaptor protein associates with T-cell receptor signal transducer SLP-76 ⁴⁶		2.1 3.0
GIT1	GPCR kinase interacting protein. Substrate and facilitator for c-src-mediated responses in vascular SMC ³⁴		2.1
HIF1- α	Activated in macrophages stimulated by oxidized low-density lipoprotein ²⁵	1.5	2.0
JAM-1	EC-associated modulator of tight junctions affecting vascular permeability ⁴⁵		2.2
JNK	Signaling activator of c-jun, inducer of apoptosis/cell growth ⁴⁴	JNK-2 1.5 JNK-1 1.0	2.5 2.1
L-caldesmon	Contractile protein increased during dedifferentiation of SMC ⁴⁷		0.3
c-src	Signaling molecule that has an important role in mediating SMC and EC growth ³⁶	2.0	3.5
TNF- α	Inflammatory cytokine associated with elevated expression in tissue and serum of patients with severe atherosclerosis ²³	1.5	3.3
TOPO-II- α	Complexes with cell cycle regulators, affecting chromatin condensation, and is a strong inhibitor of apoptosis ⁴⁰	1.3 Cleaved 1.5	2.0 2.8
TRAF4	TNFR interacting factor binds to and activates TNFR inducing inflammation ³¹	1.5	3.5

Histology

Sections (5 μ m) were stained with hematoxylin and eosin. Individual plaques were defined according to Stary's classification,¹ and features including rupture, intraplaque hemorrhage, vascularity, and lipid content were identified.

Protein Microarray Analysis

Extraction, labeling, and analysis were based on the method supplied by BD Biosciences (Clontech). Briefly, tissue was homogenized directly into extraction/labeling buffer (supplied) and centrifuged, and the protein concentration of the supernatant was measured using the Bio-Rad assay kit. Individual plaque lysates were diluted to produce a protein concentration of 1.1 mg/ml, and three pooled lysates (1 ml) were prepared, each containing equal protein quantities (250 μ g) from four separate plaque samples as follows: A) four stable arteries from nonsymptomatic patients and B) four unstable arteries from symptomatic patients. Pooled samples were divided into two equal portions, and the primary amines of one portion of each were labeled with Cy3-NHS and the other with Cy5-NHS fluorescent dyes (90 minutes/4°C). Unbound dye was removed by passing the samples through PD-10 columns, after which the protein content of the resulting labeled samples was measured. Slide mixtures were prepared containing 100 μ g of Cy3-labeled sample A + 100 μ g Cy5-labeled sample B or vice versa. Slide mixtures (20 μ g) were then incubated with pre-prepared slides for 30 minutes at room temperature with constant agitation. After washing, slides were dried by centrifugation and scanned using an Axon Systems, 4200 microarray scanner.

Data analysis was performed using GenePix Pro software. The advantages of this technique are as follow. The antibodies are applied in duplicate to the array, and the import and analyses method calculates readings based on a mean of fluorescent readings from both spots, thereby reducing the chances of assigning false-positive and false-negative results. 2) Internally normalized ratios (INRs) are produced based on results from a combination of the two slides. Hence, allowances are made for variability between Cy3 and Cy5 labeling efficiencies. INR is defined as follows:

$$\text{INR} = \frac{\text{A-Cy5/B-Cy3}}{\text{B-Cy5/A-Cy3}} \text{ relative fluorescence}$$

Average values based on duplicates that differ by more than 30% were considered invalid. Antigens were considered more abundant in sample A when $\text{INR} \geq 1.5$ and more abundant in sample B when $\text{INR} \leq 0.5$. Expression of proteins considered to be deregulated and relevant to this study were confirmed by Western blotting using the original labeled protein mixtures.

Western Blotting

A protein lysate containing an equivocated mixture of four normal carotid artery lysates was prepared, and Western blotting was used to compare protein expression from this mixture with those in samples A (stable) and B (unstable). Briefly, equal quantities of protein (15 μ g) were mixed with 2 $\times$ Laemmli sample buffer, mixed by vortexing, and boiled in a water bath for 15 minutes. Samples were separated, along with prestained molecular weight markers (32 to 200 kd), by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were elec-

Figure 2. A: Western blot showing GADS (34 kd) expression in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4 and 6); and unstable hemorrhagic/ulcerated arteries (lanes 5 and 7–9). Increased expression was seen in the latter. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemical localization of GADS showed weak staining in fibrous regions (**i**, patient 29), increased expression in macrophages from unstable regions (**ii** and **iii**, **arrows**; patients 29 and 65, respectively), and association with ECs from hemorrhagic regions (**iv**, **arrows**; patient 29). Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–iv**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

Figure 1. A: Western blot showing caspase-9 (46 kd) and cleaved caspase-9 (34 kd) expression in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4 and 6); and unstable hemorrhagic/ulcerated arteries (lanes 5 and 7–9). Increased expression was seen in the latter. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemical localization of caspase-9 in fibrotic portions of artery showing weak staining (**i**, patient 58), and in unstable plaques, showing overexpression in macrophages (**ii**, **arrow**; patient 52), SMCs (**iii**, **arrow**; patient 3), and ECs (**iv**, **arrows**; patient 3) from hemorrhagic regions. **v** is a double-labeled section showing caspase-9 association with macrophages (patient 78). Macrophages (anti-CD68) were stained with Vector blue. Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–v**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

troblotted (Hoefer, Bucks, UK) onto nitrocellulose filters (1 hour), and the filters were blocked for 1 hour at room temperature in Tris-buffered saline (TBS)-Tween (pH 7.4) containing 1% bovine serum albumin. Nitrocellulose filters were then stained with primary antibodies directed toward TNF- α , c-src, TRAF4, GIT1, TOPO-II- α , JAM-1, caspase-9, Gads, L-caldesmon, and JNK, diluted 1:1000 in the same blocking buffer, overnight at 4°C on a rotating mixer. After washing (5 $\times$ 10 minutes in TBS-Tween at room temperature), filters were stained with either goat anti-rabbit or rabbit anti-mouse horseradish peroxidase-conjugated secondary antibodies diluted in TBS-Tween containing 5% defatted milk (1:1000, 1 hour at room temperature) with continuous mixing. After a further five washes in TBS-Tween, proteins were visualized using enhanced chemiluminescence or ECL Plus chemiluminescent detection. Specificity of antibodies was confirmed by application of positive control purified cell proteins, and/or preincubation with inhibitory peptides, which abolished antibody staining (data not included).

Selected antibodies were used to examine protein expression in another 12 individual carotid plaque specimens compared with three normal arteries obtained at transplant. Blots were also stained with anti- α -actin antibodies as a protein loading control, anti-CD105 (EC marker), and anti-SMC actin (SMC marker) to determine the concentrations of individual cellular component within the plaques.

Semiquantitative analysis of protein concentration from Western blots was performed using a scanning densitometer (LKB, Bucks, UK). Results accompanying the figures are given as numerical increase or decrease compared with the control untreated cells, assigned an arbitrary optical density of 1.0. Notable increases were designated >1.5-fold and reductions <0.5-fold compared with the mean values derived from normal carotid arteries. The intensity of staining between different gels could not be compared because of variation in enhanced chemiluminescence development time. All experiments were repeated at least twice, and a representative example is shown.

Immunohistochemistry

Paraffin-processed sections were deparaffinized and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and treated with 3% hydrogen peroxide in methanol (10 minutes at room temperature) to remove endogenous peroxidase activity. Sections were placed in Triton-X 100 for 20 minutes before blocking with goat antiserum (30 minutes at room temperature). Primary antibodies were then added (TRAF4, 1:100; GIT1, 1:75; c-src, 1:100; TOPO-II- α , 1:100; JAM-1, 1:100;

caspase-9, 1:75; Gads, 1:100; and JNK, 1:75) to goat serum [diluted 1:200 in phosphate-buffered saline (PBS)], and sections were incubated overnight at 4°C. After rinsing in PBS, standard Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories) was applied, and the slides were incubated at room temperature for a further 30 minutes. Color was developed using diaminobenzidine and sections counterstained with hematoxylin before dehydration, clearing, and mounting. Specificity of the immunoreaction was confirmed by a lack of immunostaining in control sections, in which the primary antibody was replaced with either PBS or preimmune serum, and the secondary antibody was replaced by an irrelevant antibody or PBS (data not included).

Double Labeling

For double labeling after primary antibody staining and development, sections were washed in PBS, and a second antibody was applied followed by addition of alkaline phosphatase-labeled polymer and visualization with Vector Blue substrate. ECs were identified by staining with anti-CD105, SMCs using anti-SMC-actin, and macrophages by anti-CD64 antibodies.

Results

Microarray Analysis

Expression of 512 proteins was compared between crude cell protein lysates in pooled fibrous, nonulcerated stable plaques from asymptomatic patients ($n = 4$) and ulcerated, hemorrhagic unstable plaques from symptomatic patients ($n = 4$). Analysis of results, using GenePix software and the BD Biosciences microarray analysis workbook, revealed good reproducibility between duplicate spots bound to the same antibody. Less than 0.5% showed greater than 30% variation between spots (leading to their elimination from this study). In total, 21 proteins were more highly expressed ($\text{INR} \leq 0.5$) in unstable plaques, whereas 3 were increased in stable as compared to unstable ones ($\text{INR} \geq 1.5$). Several proteins, previously shown to be overexpressed in unstable atherosclerotic disease, were notably increased in unstable plaques in our study, including TNF- α , HDJ-2 (heat shock protein-40; HSP40), and c-reactive protein-2 (CRP-2).

Western Blotting-Microarray Confirmatory Studies

For Western blot analysis, we chose proteins that were novel in regard to their expression in atherosclerotic

Figure 3. A: Western blot showing expression of GIT1 (95 kd) expression in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4–6); and unstable hemorrhagic/ulcerated arteries (lanes 7–9). Increased expression was seen in lanes 7–9. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemical localization demonstrated weak staining in normal looking and fibrous regions (**i**, patient 58) but strong staining in infiltrating macrophages from ulcerated unstable plaques with high cellular content (**ii**, arrows; patient 3; **iii**, patient 52; and **iv**, patient 28). **v** is a double-labeled section showing GIT1 association with macrophages (patient 65). Macrophages (anti-CD68) were stained with Vector blue. Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–v**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

plaques and that might be involved in modulation of inflammatory, angiogenic, proliferative, and apoptotic pathways leading to plaque destabilization. TNF- α and hypoxia-inducible factor- α (HIF1- α), which have previously been reported to be up-regulated in atherosclerotic plaques, were also identified from our microarray studies and were further analyzed as internal controls. Mixtures of proteins aliquotted from samples A and B (pooled crude protein extracts) were compared with a mixture of four normal carotid artery protein lysates obtained from patients at transplant surgery. Relative protein expression is shown in Table 3. All 11 proteins examined showed differential expression (10 increased and 1 decreased) in plaque samples compared to normal carotid artery specimens. Notable differences in expression between stable and unstable plaque mixtures (A and B) were found for TNF- α , TRAF4, Gads, GIT1, Caspase-9, c-src, TOPO-II- α , and JAM-1. Increases in expression compared to normal artery were also seen in both stable and unstable artery mixtures stained with anti-TOPO-II- α , total JNK, TRAF4, TNF- α , and HIF1- α .

Western Blotting and Immunohistochemistry on Individual Plaques

Highlighted proteins from Table 3 (above) were chosen for further analysis. Total proteins from 17 carotid plaque specimens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting was used to determine the relative expression of test proteins between normal artery ($n = 3$) and stable ($n = 5$) and unstable disease ($n = 12$) (Table 2). Immunohistochemistry was performed on paraffin-processed sections from normal artery and stable and unstable plaques to identify protein localization. There was no clear association between protein expression measured by Western blotting and plaque histological classification (ie, hemorrhagic, ulcerated, and fibrous), symptomatic/asymptomatic nature of the disease, or clinical data such as cholesterol, fibrinogen, C-reactive protein, or leukocyte concentration. The level of protein expression was not related to the total numbers of SMCs or ECs present in the plaque samples, although expression of CD105 was slightly greater in 5 of 12 hemorrhagic/ulcerated plaques, whereas SMC actin was slightly reduced in 3 of 12. However, immunohistochemistry analysis demonstrated specific cellular localization of proteins with hemorrhagic and ulcerated regions in individual plaques as described below.

Caspase-9 was increased in 15 of 17 plaque samples (3 stable and all 12 unstable; 1.5- to 6.1-fold) compared with normal coronary arteries (Figure 1A). Cleaved caspase-9 (RMM, 34 kd) was also detected in all 12 of

the unstable plaques and three of five stable ones (1.5- to 2.9-fold compared to control arteries). There was highly selective staining with this antibody, with positive areas being close to unstable regions where ulceration and hemorrhage was visible together with macrophage infiltrates (Figure 1B). Also there was abundant staining in microvascular ECs surrounding active regions. There was no staining in fibrous plaques.

Mona/Gads was not detected by Western blotting in normal artery or in fibrous, stable plaques; however, 6 of 12 unstable plaques showed overexpression of this protein (1.9- to 3.5-fold compared to control arteries; Figure 2A). Intense staining of perivascular ECs around small capillaries, in the intima of SMCs, and in macrophages was seen in hemorrhagic and ulcerated plaques (Figure 2B).

GIT1 protein expression was increased in 8 of 17 plaques (zero stable and eight unstable) compared to control arteries (1.6- to 5.5-fold; Figure 3A). This protein was almost exclusively expressed in macrophages within the areas of inflammation in ulcerated-hemorrhagic plaques (Figure 3B). A few ECs were stained, but no staining was visible in normal tissue or in fibrous plaques.

JAM-1 was expressed weakly in all three normal arteries examined, but notable increases were observed in 13 of 17 plaque samples (3 stable and 10 unstable; 1.5- to 4.8-fold compared with normal arteries; Figure 4A). Small groups of mononuclear macrophages and lymphocytes were also stained within active areas of ulcerated-hemorrhagic plaques. Weaker staining was also noted in ECs from these regions (Figure 4B). No staining was visible in normal artery or fibrous plaques.

JNK (JNK1 and two isoforms) was increased in 12 of 17 plaque samples (three stable and nine unstable) compared with normal artery (JNK1; 1.5- to 3.8-fold and JNK2; 2.0- to 3.9-fold compared with normal arteries; Figure 5A). Immunohistochemistry demonstrated increased expression of JNK in macrophages and, to a lesser degree, in ECs and SMCs (Figure 5B). Generally, there were smaller numbers of positively stained cells observed in fibrous plaques.

c-src was expressed weakly in normal artery and overexpressed in 11 of 17 plaque samples (three stable and eight unstable; 2.2- to 5.6-fold compared to normal arteries; Figure 6A). More diffuse staining was present with areas of infiltrating cells, ie, macrophages and lymphocytes, and some ECs from selected regions were strongly positive. Extracellular staining was visible across the unstable plaques (Figure 6B). No staining was visible in fibrous plaques.

Native TOPO-II- α was not detected in normal arteries but was expressed in 4 of 17 plaque tissues (two stable and two unstable; 1.8- to 2.5-fold compared with control

Figure 4. A: Western blot showing expression of JAM-1 (48 kd) protein in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4 and 6); and unstable hemorrhagic/ulcerated arteries (lanes 5 and 7–9). Increased expression can be seen in unstable arteries. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemistry showed weak staining in fibrotic areas (**i**, patient 29) and increased expression in macrophages (**ii**, **arrows**; patient 40) and ECs (**iii** and **iv**, **arrows**; patient 54) from unstable hemorrhagic and ulcerated regions. **v** is a double-labeled section showing JAM-1 association with ECs (patient 65). ECs (anti-CD105) were stained with Vector blue. Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–v**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

Figure 5. A: Western blot shows expression of JNK (JNK1, 43 kD; JNK2, 51 kD) in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4–6); and unstable hemorrhagic/ulcerated arteries (lanes 7–9). Increased expression of both JNK1 and JNK2 was seen in stable and unstable plaques (lanes 4–9) compared with control arteries (lanes 1–3). In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemistry showed weak staining of SMCs in fibrous arteries (i, patient 121) but was strongly expressed by macrophages (ii, arrows; patient 65) and SMCs (iii, arrows; patient 65) in ulcerated and hemorrhagic plaques. Staining was performed using the VectaStain ABC kit. Original magnifications (**B, i–iii**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

Figure 6. A: Western blot shows expression of c-src in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4 and 6); and unstable hemorrhagic/ulcerated arteries (lanes 5 and 7–9). Increased expression of c-src was seen particularly in unstable plaques (lanes 5 and 7–9) compared with control arteries. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemistry demonstrated weak staining of c-src in fibrous regions (**i**, patient 28), and increased immunoreactivity associated with ECs (**arrows**) and macrophages (**broken arrows**) from unstable plaque regions (**ii**, **arrows**; patient 52; **iii** and **iv**, **arrows**; patient 54). Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–iv**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

arteries). The truncated form (75 kd) was detected at low levels in normal artery but was overexpressed in all 17 plaque specimens (2.0- to 3.5-fold compared with normal arteries; Figure 7A). Immunohistochemistry demonstrated strong staining of proliferating ECs and SMCs

within areas of neovascularization, from inflammatory regions containing high concentrations of cell infiltrates, in both hemorrhagic and ulcerated plaques (Figure 7B). Very weak staining was observed across stable, nonvascularized fibrous plaques.

Figure 7. A: Western blot shows expression of native Topo-II- α (170 kD) in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4–6); and unstable hemorrhagic/ulcerated arteries (lanes 7–9). Increased expression of Topo-II- α was seen in the majority of plaques, and the presence of the cleaved protein (70 kD) can be seen in **lanes 6 and 7**. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemistry showed weak staining in fibrous plaques (**i**, patient 28) and increased immunoreactivity in ulcerated plaques, particularly associated with ECs (**arrows**) and SMCs (**broken arrows**) (**ii** and **iii**, **arrows**; patient 28). **iv** is a double-labeled section showing Topo-II- α association with SMCs (patient 78, **arrows**). SMCs (anti-SMC-actin) were stained with Vector blue. Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–v**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

TRAF4 was identified and overexpressed in 13 of 17 plaque specimens (3 stable and 10 unstable) compared to normal artery (1.5- to 9.7-fold; Figure 8A). Normal

looking and fibrous arterial tissue was negative for TRAF4. In unstable (ulcerated and hemorrhagic) plaques there was strong staining within microvascular ECs in

areas of neovascularization, especially close to sites of rupture. Infiltrating macrophages and lymphocytes from these areas were also strongly stained. Plaques with the highest cellular component showed the greatest number of positive cells (Figure 8B). There was no staining in fibrous plaques.

Discussion

In this study, we have used microarray technology to investigate differences in the expression of more than 500 proteins between normal carotid arteries and those obtained from patients with stable and unstable atherosclerotic plaques. Proteomic methodologies such as protein microarrays and high-throughput Western analysis have the advantages over gene microarray studies of avoiding the necessity to validate gene expression profiles by real-time reverse transcriptase-polymerase chain reaction and confirming subsequent protein translation, which does not always show the expected correlation.²⁰ Although detection of proteins using these arrays is sensitive to nanogram quantities, errors and false-positives can occur because of nonspecific binding of proteins, degraded proteins, and/or Cy3/Cy5 fluorescent labels. In our experiments, these problems were overcome by use of the BD Bioscience data analysis program, which highlighted results in which the duplicate readings were notably different (>30%) and identified differences produced due to differential affinity of Cy3/Cy5 labels to specific proteins. Secondly, the original Cy-labeled crude protein lysates were used to confirm differential expression of relevant proteins by Western blotting. Martinet and colleagues²⁰ found a high rate of false-positive results (>50%) using high-throughput Western array technology, and the effectiveness of our initial screen was reliant on the quality of all of the individual antibodies. Therefore, the presence of weakly reacting or nonspecific antibodies could have led to some false-negative or -positive results. However, several important studies have used BD Biosciences protein antibody microarrays to great effect to dissect tumor cell signaling pathways,²¹ as well as muscular atrophy-associated proteins,²² thus validating the usefulness of these arrays. In our study, all 11 proteins identified from the original array showed differential expression between the stable and unstable plaque test mixtures, suggesting a high level of specificity of the array antibodies and a potential use for this type of proteomic analysis in accurate differential identification of plaque pathogenesis. One limitation of this work was the overall numbers of proteins studied. Hence, potentially important mediators of plaque development may have been missed. Future advances in proteomics and development of larger protein microarrays, when applied to carotid artery disease, should enable the development of a more complete understanding of the pathobiology of unstable plaque formation.

BD Bioscience protein microarrays contain duplicate printed antibodies raised against numerous proteins associated with inflammatory, pro/anti-apoptotic, and angiogenesis pathways. Our study is the first to compare

global protein expression between stable and unstable carotid artery disease. Apart from the identification of a number of novel proteins in atherosclerotic plaques, we have further validated our microarray results by confirmation of several key proteins, already established as performing a role in development of this disease. These included TNF- α ,²³ c-reactive protein-2 (CRP-2),²⁴ HIF1- α ,²⁵ and HDJ-2 (HSP40)²⁶ (data not included). Because of the relatively large quantities of protein required for these arrays, we have used pooled samples in our microarray experiments. Because only a proportion of the arterial tissue contained plaque and not all plaques will over/underexpress the same proteins, some weaker protein changes (particularly those occurring in only some of the pooled samples) could have been missed. This may also explain why the fold differences were relatively small. Further studies will use laser capture microdissection technology to dissect out individual plaque components, allowing evaluation of the proteins described here as well as other plaque-specific genes/proteins suitable for examination using gene microarrays and proteomics (using SELDI mass spectrometry), respectively.

One of the critical stages of atherosclerotic plaque development involves changes in the rates of both SMC and EC proliferation and apoptotic cell death. Identification of novel modulators of these processes should help to improve our understanding of the molecular mechanisms responsible for determining the causes of plaque thrombosis. An increase in the rate of cellular apoptosis can result in thinning of the fibrous cap, increased volume of acellular (weak) tissue, and EC hemorrhage, as described earlier. Our results demonstrate increased expression of caspase-9, JNK, and TRAF4, which could be novel promoters of apoptosis.

Western blotting demonstrated increased expression of procaspase-9 in all 12 of the unstable plaques examined, together with three of five stable ones. Increased expression of cleaved (active) caspase-9 was also detected in these samples. Immunohistochemical analysis showed that caspase-9 was localized around unstable plaque regions, particularly in association with microvascular ECs surrounding active regions. Caspase-9 is one of the initiator caspases responsible for mitochondrion-dependent apoptosis after formation of the apoptosome and could be a key determinant in the development of hemorrhagic microvessels associated with unstable plaque tissue.²⁷ Furthermore, we identified expression of caspase-9 in macrophages from the same regions. Increased apoptosis of macrophages may be a key indicator of lesional instability and plaque rupture, for example after intracellular accumulation of cholesterol.²⁸

TRAF4 is involved in p53-mediated proapoptotic signaling.²⁹ Overexpression of TRAF4 induced apoptosis in temperature-dependent cell lines (Vm10, M3) in association with activation of p53. TRAF4 has also been identified as a binding partner for p47^{phox} in ECs, whereas co-expression of TRAF4 and p47^{phox} led to JNK activation and an increase in the production of oxidants.³⁰ The results presented here, demonstrated increased expression of TRAF4 in 10 of 12 unstable plaques examined. Immunohistochemistry showed TRAF4-positive cells

were mainly microvessel ECs from ulcerated and hemorrhagic regions. Macrophages were also positively stained. Generally, TNF- α -induced inflammatory responses occur in vascular regions exposed to disturbed flow and activate MAP kinase pathways (JNK and p38) after TNF-receptor binding to TRAFs.³¹ TRAF4 may be a novel mediator of EC and macrophage apoptosis, creating destabilized areas of plaque tissue.

Various models of atherosclerosis and cell activation have demonstrated an important role for JNK activation in SMC apoptosis¹⁶ and EC mitogenesis, for example, via TNF- α -induced TRAF-SEK1 signaling pathways.³² Expression of JNK was increased in the majority of plaque samples that we examined. Increased expression of JNK could enhance blood vessel formation in hemorrhagic areas, whereas overexpression in SMCs could increase the rate of apoptosis, thus contributing to development of unstable regions. It was recently demonstrated that atherosclerosis-prone ApoE^{-/-} mice lacking JNK2 developed less atherosclerosis than the controls, and macrophages lacking JNK2 displayed suppressed foam cell formation.³³ Pharmacological inhibition of JNK efficiently reduced plaque formation in this murine model, suggesting that JNK may have an important role in development of unstable atherosclerosis via macrophage activation.

Cell signaling molecules c-src and GIT1 were also overexpressed in both ECs and macrophages from unstable hemorrhagic and ulcerated regions. GIT1 is a novel scaffold protein, recently shown to facilitate c-src-mediated MEK1 activation in a variety of cells and thus to mediate mitogenesis.³⁴ Lipid mediators of EC proliferation, including sphingosine-1-phosphate, also appear to enhance vascular barrier function through paxillin-GIT1 interactions, suggesting a possible protective influence on protection against atherosclerosis by maintenance of vascular permeability.³⁵ Although activation of c-src occurs *in vitro*, for example, after treatment of vascular SMCs with the potent vasoconstrictor urotensin II,³⁶ we were not able to show increased expression of this protein in SMCs from unstable carotid plaque regions, although the activation status of c-src was not determined.

JAM-1 is an immunoglobulin superfamily (IgSF) transmembrane receptor that can be expressed by blood platelets, ECs, epithelial cells, macrophages, and neutrophils. It is localized at EC-cell tight junctions and has an important role in modulating vascular homeostasis and leukocyte/monocyte transmigration across ECs. It can promote FGF-2-induced EC migration on vitronectin through intracellular signaling involving $\alpha_v\beta_3$ integrin.³⁷ Blocking of the extracellular domain of JAM-1 inhibits FGF-2-induced proliferation and angiogenesis of human umbilical vein ECs.³⁸ We saw a dramatic up-regulation of this protein in macrophages and also associated with

blood vessels in hemorrhagic and ulcerated plaque regions. Blocking anti-JAM-1 antibodies were able to inhibit monocyte filtration and attenuate cytokine-induced meningitis in mice.³⁹ These results suggest that pharmacological inhibition of JAM-1 expression might be of benefit in patients with atherosclerotic plaques by reducing the infiltration of macrophages.

TOPO-II- α is a cell-cycle-related marker normally found in the nucleus of cells and required for chromosome segregation during mitosis. Topoisomerase inhibitors are strong inducers of apoptosis, whereas up-regulation of TOPO-II- α gene expression results in increased mitosis and cell proliferation.⁴⁰ A proteolytically cleaved (70 kd) form of Topo-II- α has been shown in both the membrane and the nucleolus of cells undergoing necrosis and apoptosis.⁴¹ In our study, increased expression of cleaved TOPO-II- α was seen by Western blotting in the majority of plaque samples, whereas the native protein was also increased in several plaques compared with normal artery. Immunohistochemistry revealed an increase in expression of TOPO-II- α in both ECs and SMCs from hemorrhagic and ulcerated regions of unstable plaques, in particular, in regions undergoing neovascularization. One study has revealed a strong anti-proliferative effect of Topotecan (a potent TOPO-I inhibitor) on coronary vascular SMCs even in the presence of growth factors. These data suggests that topoisomerases may have a role in induction or maintenance of SMC and EC proliferation associated with arterial plaque development.⁴²

Mona/Gads is a Grb2-related Src homology 3 (SH3) and SH2 domain-containing adapter protein, expression of which is restricted to cells of hematopoietic lineage, in particular monocytes and T lymphocytes. Very little is known about the involvement of Gads in disease progression, and it has not previously been identified in association with atherosclerotic lesions. It has been shown that Gads is induced during monocyte/macrophage differentiation after interaction with macrophage colony-stimulating factor receptor (M-CSFR),⁴³ making it a potential mediator of macrophage differentiation in atherosclerosis. Our results demonstrated a strong up-regulation of this protein in macrophage infiltrates from inflammatory regions of unstable hemorrhagic and ulcerated plaques. Increased Gads expression may therefore be an important mediator of monocyte differentiation, occurring during unstable plaque development.

In conclusion, using protein microarray technology, we have identified proteins overexpressed in developing arterial plaques, in particular in association with ECs and SMCs from unstable hemorrhagic ulcerated regions. Their role in development of this disease is currently being studied. Our follow-up studies will aim to determine

Figure 8. A: Western blot shows expression of TRAF4 in normal arteries obtained at transplant (lanes 1–3); stable, fibrous arteries (lanes 4 and 6); and unstable hemorrhagic/ulcerated arteries (lanes 5 and 7–9). Increased expression of TRAF4 was seen in unstable plaques compared with control arteries and stable plaques. In the bar charts, **left** represents a comparison against α -actin (loading control), **right** is with SMC actin, and **middle** versus EC content (anti-CD105). The mean value for the three normal arteries is presented as N. **B:** Immunohistochemistry showed weak staining of TRAF4 in fibrotic areas of arteries (**i**, patient 29) but strong expression in blood vessels (**arrows**) and infiltrating lymphocytes and macrophages (**broken arrows**) from unstable hemorrhagic plaques (**ii** and **iii**, **arrows**; patient 40; **iv**, **arrows**; patient 54). **v** is a double-labeled section showing TRAF4 association with ECs (patient 78, **arrows**). ECs (anti-CD105) were stained with Vector blue. Staining was performed using the VectaStain ABC kit. Original magnifications (**B**, **i–v**): $\times 10$ (**left**); $\times 40$ (**middle**); and $\times 100$ (**right**).

their involvement in EC and SMC activation and/or survival and to identify associated cellular signaling mechanisms. Understanding the molecular mechanisms responsible for initiation of mitogenesis, angiogenesis, and apoptosis should provide the basis for development of new therapeutic treatments, perhaps using gene transfection technology to prevent or slow the rate of progression of this disease. The results presented in this study are preliminary findings, and although we have confirmed that these proteins are overexpressed by individual cells within unstable plaques, the role of these proteins is speculative, being based on their known functions within other cell systems. Our ongoing studies using primary ECs and SMCs are attempting to characterize the effects of this protein overexpression on cell survival, activation, and angiogenesis, as well as the signal transduction mechanisms associated with these processes. We anticipate that future work might examine the role of these proteins in development of atherosclerosis using *in vivo* models. Noninvasive diagnosis and prognosis of patients with the highest risk of developing symptomatic disease would represent an important advancement in our ability to reduce the incidence of coronary heart disease and stroke. Novel proteins identified here might be useful serum markers of disease status and could form part of a future protein microarray panel used to identify patients at risk of thrombosis as well as to predict treatment outcome and follow-up.

References

- Hennerici MG: The unstable plaque. *Cerebrovasc Dis* 2004, 17:17–22
- Moulton KS: Plaque angiogenesis: its function and regulation. *Cold Spring Harbor Symp Quant Biol* 2002, 11:471–482
- DeGraba TJ, Siren AL, Penix L, McCarron RM, Hargraves R, Sood S, Pettigrew KD, Hallenbeck JM: Increased endothelial expression of intercellular adhesion molecule-1 in symptomatic versus asymptomatic human carotid atherosclerotic plaque. *Stroke* 1998, 29:1405–1410
- Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, Thompson MM: Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. *Stroke* 2000, 31:40–47
- Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A: Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation* 2001, 104:1598–1603
- Elkind MS, Cheng J, Boden-Albala B, Rundek T, Thomas J, Chen H, Rabbani LE, Sacco RL: Tumor necrosis factor receptor levels are associated with carotid atherosclerosis. *Stroke* 2002, 33:31–37
- Brocheriou I, Stengel D, Mattson-Hulten L, Stankova J, Rola-Pleszczynski M, Koskas F, Wiklund O, Le Charpentier Y, Ninio E: Expression of platelet-activating factor receptor in human carotid atherosclerotic plaques: relevance to progression of atherosclerosis. *Circulation* 2000, 102:2569–2575
- Irvine CD, George SJ, Sheffield E, Johnson JL, Davies AH, Lamont PM: The association of platelet-derived growth factor receptor expression, plaque morphology and histological features with symptoms in carotid atherosclerosis. *Cardiovasc Surg* 2000, 8:121–129
- Fryer JA, Myers PC, Appleberg M: Carotid intraplaque hemorrhage: significance of neovascularity. *J Vasc Surg* 1987, 6:341–349
- Mofidi R, Crotty TB, McCarthy P, Sheehan SJ, Mehigan D, Keaveny TV: Association between plaque instability, angiogenesis and symptomatic carotid occlusive disease. *Br J Surg* 2001, 88:945–950
- Folkman J: Angiogenesis in cancer, vascular rheumatoid and other diseases. *Nat Med* 1995, 1:27–31
- O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE: Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation* 1996, 93:672–682
- Woodside KJ, Hernandez A, Smith FW, Xue XY, Hu M, Daller JA, Hunter GC: Differential gene expression in primary and recurrent carotid stenosis. *Biochem Biophys Res Comm* 2003, 302:509–514
- Choy JC, Granville DJ, Hunt DW, McManus BM: Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 2001, 33:1673–1690
- Saxena A, McMeekin JD, Thomson DJ: Expression of Bcl-x, Bcl-2, Bax and Bak in endarterectomy and atherectomy specimens. *J Pathol* 2002, 196:335–342
- Metzler B, Hu YH, Dietrich H, Xu QB: Increased expression and activation of stress-activated protein kinases/c-jun NH2-terminal protein kinases in atherosclerotic lesions coincide with p53. *Am J Pathol* 2000, 156:1875–1886
- Martinet W, Schrijvers DM, De Meyer GRY, Thielmans J, Knaapen MW, Herman AG, Kockx MM: Gene expression profiling of apoptosis-related genes in human atherosclerosis: up regulation of death-associated protein kinase. *Arterioscler Thromb Vasc Biol* 2002, 22:2023–2029
- Haley KJ, Lilly CM, Yang JH, Feng Y, Kennedy SP, Turi TG, Thompson JF, Sukhova GH, Libby P, Lee RT: Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* 2000, 102:2185–2189
- McCaffrey TA, Fu C, Du B, Eksinar S, Kent KC, Bush Jr H, Kreiger K, Rosengart T, Cybulsky MI, Silverman ES, Collins T: High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J Clin Invest* 2000, 105:653–662
- Martinet W, Schrijvers DM, De Meyer GRY, Herman AG, Kockx MM: Western array analysis of human atherosclerotic plaques: down regulation of apoptosis-linked gene 2. *Cardiovasc Res* 2003, 60:259–267
- Yamagiwa Y, Marienfeld C, Meng F, Holcik M, Patel T: Translational regulation of X-linked inhibitor of apoptosis protein by interleukin-6: a novel mechanism of tumor cell survival. *Cancer Res* 2004, 64:1293–1298
- Anderson K, Potter A, Baban D, Davies KE: Protein expression changes in spinal muscular atrophy revealed with a novel antibody array technology. *Brain* 2003, 126:2052–2064
- Mizia-Stec K, Gasior Z, Zahorska-Markiewicz B, Janowska J, Szulc A, Jastrzebska-Maj E, Kobielusz-Gembala I: Serum tumor necrosis factor-alpha, interleukin-2 and interleukin-10 activation in stable angina and acute coronary syndromes. *Coron Artery Dis* 2003, 14:431–438
- Zairis MN, Manousakis SJ, Stefanidis AS, Vitalis DP, Tsanis EM, Hadjigeorgiou SM, Fakiolas CN, Pissimissis EG, Olympios CD, Fousas SG: C-reactive protein and rapidly progressive coronary artery disease—is there any relation? *Clin Cardiol* 2003, 26:85–90
- Shatrov VA, Sumbayev VV, Zhou J, Brune B: Oxidized low-density lipoprotein (oxLDL) triggers hypoxia-inducible factor-1alpha (HIF-1alpha) accumulation via redox dependent mechanisms. *Blood* 2003, 101:4847–4849
- Nguyen TQ, Jaramillo A, Thompson RW, Dintzis S, Oppat WF, Allen BT, Sicard GA, Mohanakumar T: Increased expression of HDJ-2 (hsp40) in carotid artery atherosclerosis: a novel heat shock protein associated with luminal stenosis and plaque ulceration. *J Vasc Surg* 2001, 33:1065–1071
- Chen M, Wang J: Initiator caspases in apoptosis signaling pathways. *Apoptosis* 2002, 7:313–319
- Yao PM, Tabas I: Free cholesterol loading of macrophages is associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway. *J Biol Chem* 2001, 276:42468–42476
- Sax JK, El-Deiry WS: Identification and characterization of the cytoplasmic protein TRAF4 as a p53-regulated proapoptotic gene. *J Biol Chem* 2003, 278:36435–36444
- Xu YC, Wu RF, Gu Y, Yang YS, Yang MC, Nwariaku FE, Terada LS: Involvement of TRAF4 in oxidative activation of c-Jun N-terminal kinase. *J Biol Chem* 2002, 277:28051–28057
- Yamawaki H, Lehoux S, Berk BC: Chronic physiological shear stress inhibits tumor necrosis factor-induced proinflammatory responses in rabbit aorta perfused in vivo. *Circulation* 2003, 108:1619–1625

32. Yoshizuma M, Fujita Y, Izawa Y, Suzaki Y, Kyaw M, Ali N, Tsuchiya K, Kagami S, Yano S, Sone S, Tamaki T: Ebselen inhibits tumor necrosis factor- α -induced c-Jun N-terminal kinase activation and adhesion molecule expression in endothelial cells. *Exp Cell Res* 2004, 292:1–10
33. Ricci R, Sumara G, Rozenberg I, Kurrer M, Akhmedov A, Hersberger M, Eriksson U, Eberli FR, Beacher B, Boren J, Chen M, Cybulsky MI, Moore KJ, Freeman MW, Wagner EF, Matter CM, Luscher TF: Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. *Science* 2004, 306:1558–1561
34. Yin G, Haendeler J, Yan C, Berk BC: GIT1 functions as a scaffold for MEK1-extracellular signal regulated kinase 1 and 2 activation by angiotensin II and epidermal growth factor. *Mol Cell Biol* 2004, 24:875–885
35. Shikata Y, Birukov KG, Garcia JGN: S1P induces FA remodelling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. *J Appl Physiol* 2003, 94:1193–1203
36. Watanabe T, Pakala R, Katagiri T, Benedict CR: Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation* 2001, 104:16–18
37. Naik MU, Mousa SA, Parkos CA, Naik UP: Signaling through JAM-1 and $\alpha_v\beta_3$ is required for the angiogenic action of bFGF: dissociation of the JAM-1 and $\alpha_v\beta_3$ complex. *Blood* 2003, 102:2108–2114
38. Naik MU, Vuppalanchi D, Naik UP: Essential role of junctional adhesion molecule-1 in basic fibroblast growth factor-induced endothelial cell migration. *Arterioscler Thromb Vasc Biol* 2003, 23:2165–2171
39. Del Maschio A, De Luigi A, Martin-Padura I, Brockhaus M, Bartfai T, Fruscella P, Adorini L, Martino G, Furlan R, De Simoni MG, Dejana E: Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). *J Exp Med* 1999, 190:1351–1356
40. Sordet O, Khan QA, Kohn KW, Pommier Y: Apoptosis induced by topoisomerase inhibitors. *Curr Med Chem Anti-Cancer Agents* 2003, 3:271–290
41. Endo H, Ishimura N, Tanaka Y, Yamashita M, Sakaki M, Hayashi Y, Sano T: Unique cell membrane expression of topoisomerase-II α as a useful diagnostic marker of liposarcoma. *Pathol Int* 2004, 54:145–150
42. Brehm BR, Bock C, Wesselborg S, Pfeiffer S, Schuler S, Schulze-Osthoff K: Prevention of human smooth muscle cell proliferation by the topoisomerase I inhibitor topotecan. *Biochem Pharmacol* 2001, 61:119–127
43. Bourgin C, Bourette RP, Arnaud S, Liu Y, Rohrschneider LR, Mouchiroud G: Induced expression and association of the Mona/Gads adapter and Gab3 scaffolding protein during monocyte/macrophage differentiation. *Mol Cell Biol* 2002, 22:3744–3756
44. Hoefen RJ, Berk BC: The role of MAP kinases in endothelial activation. *Vasc Pharmacol* 2002, 38:271–273
45. Dejana E, Spagnuolo R, Bazzoni G: Interendothelial junctions and their role in the control of angiogenesis, vascular permeability and leukocyte transmigration. *Thromb Haemost* 2001, 86:308–315
46. Harkiolaki M, Lewitzky M, Gilbert RJ, Jones EY, Bourette RP, Mouchiroud G, Sondermann H, Moarefi I, Feller SM: Structural basis for SH3 domain-mediated high-affinity binding between Mona/Gads and SLP-76. *EMBO J* 2003, 22:2571–2582
47. Lijima M, Yamamoto J, Takada N, Ohata H, Momose K: Changes in Ca^{2+} signaling and contractile protein isoforms in smooth muscle cells from guinea pig ileum during culture. *J Smooth Muscle Cell Res* 2001, 37:53–66