2.3. Primers and probes for real-time RT-PCR

Using Primer Express™ software (Applied Biosystems, Foster, CA), primers were designed for each of the genes for MMP-1, -2, -3 and -9, TFPI-2 and TIMP-1, -2 and -3, and the TaqMan probe inherent to each primer set was prepared, which was an oligonucleotide labeled with a reporter dye (FAM) at the 5′-end and a quencher dye (TAMRA) at the 3′-end. The sequences of the primers and TaqMan probes of MMPs-1, -2, -3, -9, TIMPs-1, -2 and -9 were reported elsewhere [13], and those for TFPI-2 were SENSE = CGATGCTTGCTGGAGGATAGA; ANTISENSE = ACACTGGTCGTCCACACTCACT; Taqman probe = 5′-FAM-AAGTTCCCAAAGTTTGCCGGCTGC-TAMRA-3′; TFPI-2 SENSE = CGATGCTTGCTGGAGGATAGA; ANTISENSE = ACACTGGTCGTCCACACTCACT; Taqman probe = 5′-FAM-AAGTTCCCAAAGTTTGCCGGCTGC-TAMRA-3′.

Real-time RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction solution was assembled in a volume of 25 μl, which comprised TaqMan Universal PCR Master Mix (Applied Biosystems), forward and reverse primers (. nal concentration 300 nM each), TaqMan probe (. nal concentration 200 nM) and cDNA mixture (about 2.5 ng). Throughout this study, the cDNA mixture from a particular sample was used to generate the working standard for quantitation of the cDNA of interest, which plots the relationship between the dilution of the standard cDNAs and the corresponding C_t value (the number of cycles necessary to obtain a threshold . uorescent signal) [13]. The initial quantity of the cDNA of interest in a certain cDNA mixture was calculated from the working standard and then normalized to that of GAPDH determined with TaqMan Assay Reagent Endogenous Control™ (Applied Biosystems). The normalized value for each target cDNA re. ects the expression level of the corresponding gene in a test sample relative to the standard tissue. The accuracy of the present real-time RT-PCR for determining mRNA expression in human vascular tissue was already con. rmed by comparing the results with those determined by conventional RT-PCR method [13].

2.4. Expression and function of MMP

To determine expression and function of MMP in its protein level, carotid tissue samples from 10 patients, in whom enough amounts of proteins could be extracted, were examined by Western blotting and gel zymography. The extracted protein was separated by SDS-PAGE and blotted onto a Hybond-ECL nitrocellulose membrane (Amersham) with the use of primary (40 μg/ml) and secondary (1:2000, Amersham) antibodies. As for zymography, proteins with gelatinolytic activity were identi. ed by use of substrate gels prepared by incorporation of gelatin (1 mg/ml; Wako) into a SDS-PAGE. After elecrophoresis, gels were washed in 2.5% Triton X-100 for 30 min to remove SDS. The gel was equilibrated for 30 min at room temperature with gentle agitation then incubated for overnight at 37 °C in 50 mM Tris/HCl, pH 7.5, containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35. Gels were then . xed and stained with 0.25% Coomassie Brilliant Blue R-250 (Wako). The product of the optical net density of the band was compared with a positive control (HT-1080 human . brosarcoma cells for Western blotting and human MMP-2 and human MMP-9, 1.5 ng, CC073; CHEMICON for zymography) to obtain a ratio comparable between gels.

2.5. Histology and immunohistochemistry

A part of the plaque was placed in tissue . xative (Histochoice, Hedwin, Baltimore). After overnight . xation, the samples were paraf. n embedded and sectioned at 4-μm intervals. Tissue sections were deparaf. nized with xylene followed by immersion in graded alcohol. They were washed three times for 5 min each in phosphate-buffered saline (PBS) and blocked with bovine serum albumin for 60 min. Specimens were then incubated with primary antibodies against CD-68, MMPs, TIMPs and TFPI-2 (Fuji Chemical, Tokyo, Japan) overnight at 4 °C. After they were washed in PBS, specimens were incubated with biotinylated rabbit anti-mouse IgG for 60 min at room temperature. Specimens were then washed with PBS, stained with horseradish peroxidase-conjugated streptavidin, and . nally incubated with substrate solution for 1–15 min. The tissue sections were also stained with hematoxylin–eosin and elastica van Gieson for evaluation of plaque composition and . brous cap disruption, as described by Carr et al. [2]. Plaque was de. ned as atheromatous if the area of lipid core was ≥30% of the whole plaque area and as . brous plaque if <30% in terms of its vulnerability [14].

2.6. Data analysis

The mean and standard error of triplicate data are presented. Statistical analysis was performed by paired t-test using Stat View 5.0 software on a Macintosh computer and by Wilcoxon matched-pair signed-rank test if appropriate. A p-value <0.05 was considered signi. cant.

3. Results

3.1. Patient and plaque characteristics

Atheromatous plaque was observed in 15 samples and . brous plaque in 9 samples. Disruption of the . brous cap was observed in 11 samples with atheromatous plaque and was not observed in 13 samples, which consisted of 4 atheromatous and 9 . brous plaques. Although levels of HbA1c and LDL-cholesterol in patients with plaque disruption tended to be higher than those in patients without disruption, there was no statistically signi. cant difference in their clinical back-

Table 2
MMP, TFPI-2 and TIMP levels

Mrna	Control	Plaque	p-Value
MMP-1	0.60 ± 0.16	1.53 ± 0.25	<0.01
MMP-2	0.80 ± 0.11	0.88 ± 0.14	NS
MMP-3	0.46 ± 0.18	1.99 ± 0.59	<0.05
MMP-9	0.58 ± 0.21	2.00 ± 0.51	<0.01
TFPI-2	0.94 ± 0.23	0.32 ± 0.08	<0.01
TIMP-1	0.81 ± 0.10	1.28 ± 0.23	<0.05
TIMP-2	1.12 ± 0.15	0.95 ± 0.17	NS
TIMP-3	0.47 ± 0.16	0.67 ± 0.17	NS

ground. Also there was no difference in hs-CRP level between two patient groups, although mean value in all patients was higher than normal value (Table 1).

3.2. Expression levels of MMPs, TIMPs and TFPI-2

From removed samples with a wet weight of 11.69 ± 2.64 mg, 0.49 ± 0.22 μg total RNA was extracted for analysis. Ampli. cation of GAPDH was equivalent among all the cDNAs synthesized. Each primer set for PCR exponentially ampli. ed its target cDNA according to the cycle number. Normalized values for MMP, TIMP and TFPI-2 gene expression in plaque and adjacent control tissue (controls) are summarized in Table 2. In the plaques, the gene expression levels of MMP-1 (1.53 ± 0.25), MMP-3 (1.99 ± 0.59) and MMP-9 (2.00 ± 0.51) were signi. cantly higher than those in the controls (0.60 ± 0.16, 0.46 ± 0.18 and 0.58 ± 0.21, respectively, $p<0.05$). However, no difference was found in the expression level of MMP-2 gene between the plaques and controls (0.88 ± 0.14 versus 0.80 ± 0.11). It was quite interesting that TFPI-2 gene expression was signi. cantly higher in the controls (0.94 ± 0.23) than that in plaques (0.32 ± 0.08, $p<0.01$).

As for TIMP genes, the only TIMP-1 gene was signi. cantly upregulated in plaques in comparison with that in the controls (1.28 ± 0.23 versus 0.81 ± 0.10, $p<0.05$) (Table 2). Among the combination of the ratios of the four MMPs to the three TIMPs examined in this study, the expression ratios of MMP-1 to TIMP-1, MMP-3 to TIMP-3 and MMP-9 to TIMP-1 were signi. cantly higher in plaques than in the controls (2.98 ± 0.77 versus 0.99 ± 0.43, 2.18 ± 0.53 versus 0.63 ± 0.22 and 1.80 ± 0.14 versus 0.83 ± 0.09, respectively, $p<0.05$) (Fig. 1). Of interest, in plaques with disruption of . brous cap, MMP-9 expression (3.36 ± 1.52) and the ratio of MMP-9 to TIMP-1 (1.66 ± 0.12) were signi. cantly higher than those in plaques without disruption (1.11 ± 0.52 and 0.76 ± 0.12, respectively), although TFPI-2 gene expression was not different between these groups (0.27 ± 0.08 versus 0.40 ± 0.18).

MMP-9 protein was expressed both in disrupted and non-disrupted plaques, but was not expressed or only slightly expressed in controls. Under these conditions, net expression of MMP-9 was signi. cantly higher in disrupted (2.61 ± 0.17,

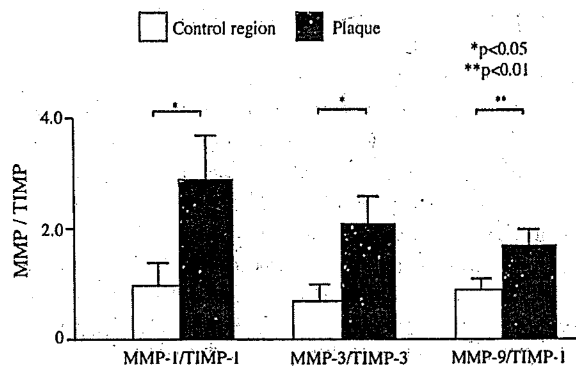

Fig. 1. Imbalanced expression of matrix metalloproteinase (MMP) to tissue inhibitor of matrix metalloproteinase (TIMP) genes in carotid plaque. Vertical axis represented the ratio of MMP/TIMP. Open columns represent values from control regions and closed columns values from plaques.

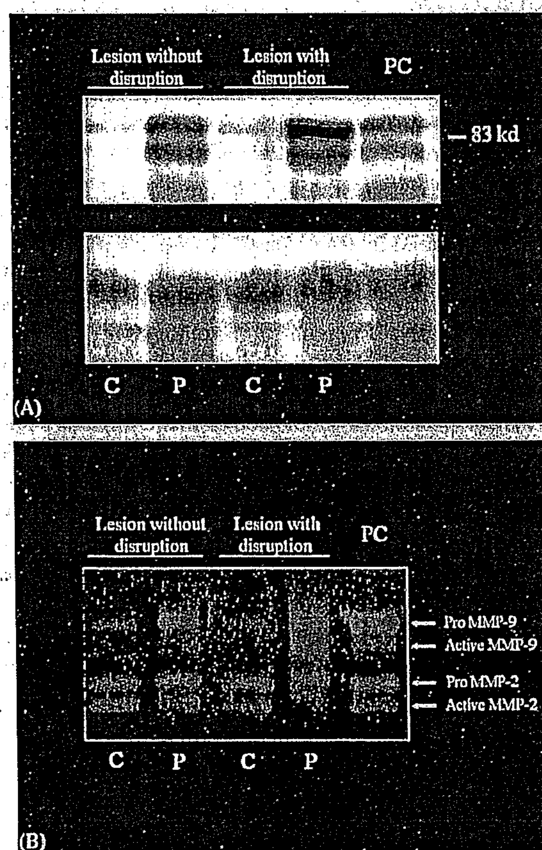

Fig. 2. Expression and function of MMP-9 in protein level. (A) Western blotting for matrix metalloproteinase MMP-9 (upper) and an internal marker protein, endothelin receptor (ETR) (lower), in non-ruptured lesion, ruptured carotid lesions and positive control (PC). MMP-9 was clearly expressed in the plaque (P) and PC, whereas in the control region (C), little expression of MMP-9 was observed. Note that both pro* and active** form of MMP-9 appears to be highly expressed in the ruptured plaque, in comparison with the non-ruptured plaque, although ETR protein is equally expressed. (B) By zymography, increased size and staining of both pro- and active forms of MMP-9 particularly in ruptured plaques, although MMP-2 activity was not different in each lane as observed in mRNA analysis.

$n=4$) than in non-disrupted plaques (1.11 ± 0.12, $n=6$, $p<0.05$), despite the equal expression of an internal marker protein, endothelin-1 receptor (Fig. 2A). Interestingly, the amount of active form MMP-9 determined by zymography was signi. cantly higher in the disrupted (2.62 ± 0.12) than in non-disrupted plaques (0.72 ± 0.07, $p<0.05$), although pro MMP-9 activity was not signi. cantly different in disrupted (1.8 ± 0.10) and non-disrupted plaques (1.4 ± 0.11) (Fig. 2B). There were no signi. cant differences between the levels of pro and active forms of MMP-2, as demonstrated in its mRNA expression.

3.3. Immunohistochemistry

In the adjacent control regions (Fig. 3A), there was mild atherosclerosis where a few CD-68 positive macrophages existed. Under these conditions, MMPs and TIMPs were scatteringly positive. In contrast, TFPI-2 was diffusely positive in the intima and media. Plaque regions mainly consisted of lipid-rich core and . brous tissue (Fig. 3B) where CD-68 positive macrophages were accumulated particularly in the shoulder regions of atheroma and all MMPs and TIMPs were strongly positive. It was interesting that, under these conditions, TFPI-2 was regionally positive in the plaque regions. Because of small number of examined plaques, we could not correlate expression of MMPs, TIMPs and TFPI-2 to the stage of plaque development.

4. Discussion

4.1. Gene expression of MMPs, TIMPs and TFPI-2 in plaque

One of the striking . ndings of the present study was that with a decreased TFPI-2 gene expression, the MMP-9 gene together with the MMP-9 protein was signi. cantly upregulated in plaques, particularly in plaques with disrupted . brous cap. Increased production of MMP-9 is thought to contribute to the progressive deterioration of the elastic lamellae associated with vessel remodeling, which could be closely related to the occurrence of plaque disruption [15]. Indeed, previous studies indicated that MMP-9 was present in the coronary plaque from unstable angina [16] and carotid plaque from

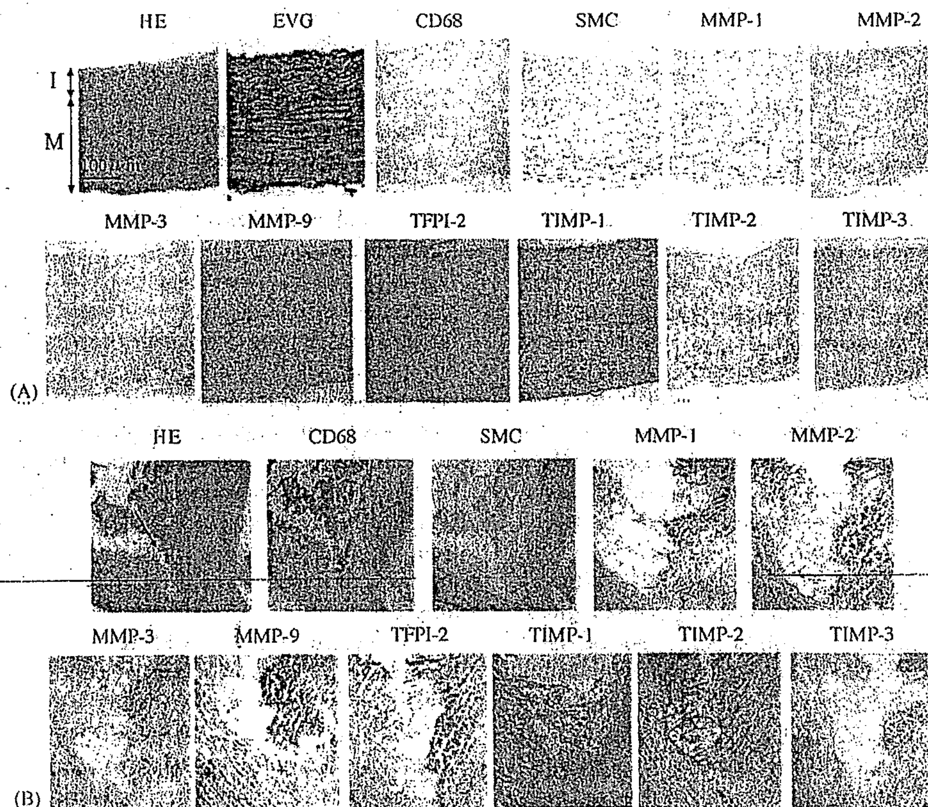

Fig. 3. Histologic and immunohistologic . ndings (with original magni. cation of ×25). (A) In the control tissues, there existed mild atherosclerotic lesion where a few CD-68 positive macrophages was found. Under these conditions, tissue factor pathway inhibitor (TFPI)-2 was diffusely positive in the intima and media, although matrix metalloproteinases (MMPs) and tissue inhibitor of MMPs (TIMPs) were scatteringly positive. An arrow indicates boundary between intima and media. (B) In plaque lesions with a lipid-rich core where CD-68 positive macrophages were accumulated, all MMPs and TIMPs were strongly positive particularly in the shoulder regions of atheroma (arrow). It was interesting that, under these conditions, TFPI-2 was regionally positive in this lesion. EVG, elastica van Gieson; HE, hematoxylin–eosin; I, intima; M, media; SMC, smooth muscle cell.

symptomatic patients [17]. We in fact demonstrated greater upregulation and function of MMP-9 in plaques with . ssured . brous cap at the mRNA as well as protein level, based on histological . ndings.

Simultaneous upregulation of the MMP-1 and -3 genes was also observed, as previously reported [18,19]. MMP-1 speci. cally cleaves collagen types I and III, which are key components of the extracellular framework of the arterial wall and major constituents of human atherosclerotic plaques, and activate other MMPs [6] that degrade denatured collagen, gelatin and elastin. MMP-3 has the widest substrate repertoire of all MMPs, showing activity against most of the extracellular proteins and proteoglycans [20]. However, unlike MMP-9, there were no differences in the expression of MMP-1 and -3 genes between plaques with and without rupture. This suggests that simultaneous upregulation of these MMPs is a plausible phenomenon in the development of atherosclerotic plaques.

This study demonstrates diminished gene expression of TFPI-2 in plaques that contain abundant MMPs. TFPI-2, originally considered as a serine proteinase inhibitor, is known to be highly expressed in smooth muscle cells of the relatively non-diseased tissue favoring ECM stability by inactivating collagenases such as MMP-1 as well as gelatinases probably through direct protein/protein interactions. Indeed, Herman et al. [7] demonstrate inverse relation between TFPI and MMP activity in atherosclerotic tissue. Thus, decreased TFPI-2 gene expression in plaques, as observed in the present study, might allow increased matrix degradation by MMP-1, -3 and -9 in plaques, enhancing their susceptibility to plaque development. It is interesting, under these conditions, TIMP-1 exhibited signi. cantly higher expression in plaques than in controls. The combined deletion of TIMP-1 and ApoE in mice leads to a reduction in atherosclerotic plaque size [21], whereas overexpression of TIMP-1 induced by adenovirus-mediated transfer in ApoE-de. cient mice leads to a decrease in plaque size and an increase in collagen content [22]. Taken together, under the condition where TFPI-2 was diminished to express, upregulation of TIMP-1 seems to counteract overexpression of MMPs, to exert an inhibitory effect on the development of atherosclerotic plaque.

However, the expression ratios of MMPs to TIMP-1 were still higher in the plaque compared with the control regions. Compensatory expression of TIMP-1 might not be suf. - cient to counteract the degenerative role of MMPs in the plaque, thus contributing to the development of atherosclerotic plaque. Particularly, the MMP-9/TIMP-1 ratio was signi. cantly higher in plaques with disruption than in those without disruption. This suggests the functional signi. cance of the imbalance of expression of these genes in the occurrence of plaque disruption. It would be of interest to examine which can play a more important role, TFPI-2 or TIMP-1, for the regulation of MMP activity, since compartmentalization might result in distinct microenvironments with corresponding variations in MMP/inhibitor ratios.

4.2. Clinical implications and limitations

A recent experimental study in which local MMP-9 was upregulated by gene transfection resulted in enhanced formation of local thrombus [23]. On the contrary, manipulation to augment expression of expression of TIMPs prevented the occurrence of plaque disruption [22]. Therefore, one might speculate that the altered balance of MMP-9/TIMP-1 with decreased TFPI-2 observed in the present study contributes to plaque disruption associated with or without regional thrombosis.

The carotid plaques examined in the present study were obtained from highly stenotic lesion probably representing the . nal stage of plaque development and destabilization. In acute coronary syndrome, however, atherosclerotic plaque disruption is known to occur at the sites of mild to moderate stenotic lesions [24] that were not examined in the present study. Although preliminary results indicate that in coronary plaques related to acute coronary syndrome MMP-9 gene was highly expressed in comparison with that in plaques from stable coronary disease [25], further study will need to con- . rm gene expression in carotid plaque from mild to moderate stenotic lesion.

The present study has a limitation regarding histological assessment of the presence of plaque disruption. Only a small portion of each plaque was examined histologically, and it may well be that features were missed in some patients. Several reports suggest that vulnerability to plaque rupture is a multifocal phenomenon particularly at the time of acute presentation [26,27]. Conversely, one might argue that we did not necessarily determine mRNA expression levels in the part of the plaque where histological analysis was performed. Even under these conditions, imbalanced expression of MMPs/TIMPs with reduction of TFPI-2 was observed in plaques, particularly in those associated with disruption. That the control regions were obtained from adjacent to the culprit lesion is another limitation. However, there was no histological evidence for plaque disruption in the control regions used for present study even in the presence of mild atherosclerosis. It can not be excluded, however, that the disruption of the . brous cap could be resulted from surgical procedure, although we carefully examined the part of plaque where surgical procedures was not affected.

Whether upregulation of MMPs is the cause or result of plaque disarrangement is unclear. A recent study suggested that MMP-9 might have a protective effect against plaque development in double ApoE and MMP-9 knockout mice [28]. Thus, a causal relationship cannot be concluded until a controlled trial with a speci. c MMP-9 inhibitor is performed. Recently, MMP-8, traditionally associated only with neutrophils, which enhanced matrix breakdown by activating MMPs and/or by inactivating TIMP-1, was found to be highly expressed in macrophages in disrupted plaques [29]. Reduced expression of TFPI-2 might be related to the enhanced expression of neutrophil elastase in plaques, although MMP-

8 gene expression was not determined in the present study.

In the present study, we used real-time RT-PCR, which gives an estimate of mRNA expression instead of protein level for each enzyme and inhibitor, because it is still dif. cult to extract some proteases such as MMP-1, which binds strongly to connective tissue and to quantitatively assay enzyme activities [30]. However, it is important to determine the activity of TIMPs in protein level, since determination of gene expression can sometimes misinterpret the actual change of protein expression [31]. Therefore, evaluation of mRNA expression of multiple genes by the present real-time RT-PCR method in combination with determination of protein should be done for systematic evaluation of the activities of MMPs, TIMPs and TFPI-2 in clinical tissue samples.

Finally, the precise mechanism of the sustained overexpression of MMPs and TIMPs with reduction of TFPI-2 in advanced atherosclerotic plaque is still unclear. Our preliminarily report indicate that CXCR-2, a chemokine receptor, gene was highly upregulated in accordance with MMP expression in macrophages [32]. This suggests that overexpression of MMPs could be related to a continuous in. ammatory reaction, although there was no difference in serum levels of hs-CRP between patients with ruptured and nonruptured plaques. Further study of the regulatory mechanisms of chemokine and cytokine systems with transcription factors that also play a crucial role in MMP expression [33] may demonstrate a signi. cant pathway for the expression and activation of proteinases and their inhibitors in human atherosclerotic lesions.

5. Conclusion

We applied a real-time RT-PCR method to quantitate mRNA expression in small samples of human carotid plaque. Levels of MMP-1, -3, -9 and TIMP-1 mRNAs were significantly upregulated in human carotid plaque where TFPI-2 mRNA was decreased to be expressed. The particular upregulation of MMP-9 and resultant imbalance of MMP-9/TIMP-1 expression could play a pivotal role in plaque disruption.

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Sustained Upregulation of Inflammatory Chemokine and Its Receptor in Aneurysmal and Occlusive Atherosclerotic Disease

—— Results From Tissue Analysis With cDNA Macroarray and Real-Time Reverse Transcriptional Polymerase Chain Reaction Methods ——

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Background Although cytokines are known to be pivotal in the development of atherosclerotic diseases, few data exist regarding their expressions in the established stages such as aneurysmal or occlusive lesions. Therefore, in the present study the gene expression levels of cytokine-related substances in abdominal aortic aneurysm (AAA) and carotid artery stenosis (CAS) were determined using cDNA macroarray and real-time reverse transcriptase polymerase chain reaction (RT-PCR) methods.

Methods and Results Tissue samples were obtained from 31 patients with AAA and 24 with CAS. The array-specific [^{33}P]-labeled cDNA probe mixture synthesized from 2.5• g total RNA with gene-specific primers was hybridized with nylon membranes containing 375 cDNA clones. Densitometric analysis confirmed differences in expression (>5-fold) for 97 of the cytokine-related gene products between AAA and adjacent control tissue. Among these, simultaneous upregulation was found in the expression of interleukin (IL)-8 (9-fold) and its receptor, CXCR-2 (11-fold). Thus, the expressions of IL-8 and CXCR-2 were further quantified by real-time RT-PCR. The expression of both the genes was significantly upregulated in both AAA and CAS compared with control regions as followed: IL-8=0.53±0.16 vs 0.11±0.04 ($p<0.01$); CXCR-2=2.04±0.75 vs 0.29±0.10 ($p<0.01$) in AAA, and IL-8=1.35±0.25 vs 0.60±0.16; CXCR-2=2.00±0.51 vs 0.58±0.21 ($p<0.05$) in CAS. Under these conditions, the gene expressions of monocyte chemotactic protein-1 and its receptor, CCR-2, were not significantly different in the control and diseased regions of both AAA and CAS.

Conclusions Sustained upregulation of IL-8 and CXCR-2 was observed in both AAA and CAS, suggesting the inflammatory process is still active in established dilated and occlusive atherosclerotic diseases. Whether upregulation of this system could be protective or not protective for disease development requires further study. (*Circ J* 2005; **69:** 1490–1495)

Key Words: Aneurysm; Atherosclerosis; CCR-2; Chemokines; CXCR-2; Interleukin-8

Upregulation of several genes relevant to the pathophysiology of dilated atherosclerotic diseases, such as abdominal aortic aneurysm (AAA), and stenotic diseases, such as carotid artery stenosis (CAS), has been demonstrated, particularly regarding enzymes of the matrix metalloproteinase (MMP) family and their endogenous inhibitors.[1] Indeed, we previously reported that in both AAA and CAS of human tissues, upregulation of MMP genes was observed in comparison with adjacent control tissues.[2,3]

In the early stage of atherosclerosis, cytokines such as monocyte chemotactic protein (MCP)-1, interleukin (IL)-6 and their receptors are overexpressed, which contributes to the initiation and development of atherosclerotic disease.[4–6] However, for the established stage of these diseases when the clinical manifestations of vessel dilation or occlusion become apparent, few data exist regarding the gene expression of cytokines or chemokines in relation to overexpression of MMPs.[7,8] Such evidence may contribute to our understanding of the role of cytokines and their receptors, protective or not protective, in the clinical course of vascular disease. In the present study, the gene expressions of cytokines and their receptors were systematically examined

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using cDNA macroarray and then quantitatively evaluated with real-time reverse transcription polymerase chain reaction (RT-PCR) methods.

Methods

Patients and Tissue Sampling

The protocol of this study was approved by the institutional committee for ethical review. Written informed consent was given by all patients. Tissue samples were obtained from 31 patients who underwent elective graft replacement for AAA with a diameter of 58±18 mm by computed tomography (29 males, 2 females; mean age, 71±2 years) and from 24 patients who underwent carotid endarterectomy for severe stenosis (>90% diameter stenosis by angiography) of the extracranial carotid artery (all males; mean age, 68±2 years). As for medical treatment, 20 patients with AAA and 10 with CAS were treated with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, and 17 with AAA and 7 with CAS were with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockade.

During graft replacement for AAA, a strip of aortic wall that contained the dilated region and relatively normal portion was carefully excised. Carotid endarterectomy was extended in a caudal direction to include a sample of minimally affected common carotid artery proximal to the plaque but in continuity with it, to act as a paired control. After removing part of the tissue for histological examination, all the samples were quickly frozen in liquid nitrogen and stored at –80°C until extraction of RNA.

RNA Preparation and cDNA Synthesis

Experimental procedures have been already described elsewhere[2] Briefly, the samples were homogenized in 1.0 ml ISOGEN™ reagent (Nippon Gene, Tokyo, Japan), thoroughly mixed with 0.2 ml chloroform and centrifuged at 15,000 G for 15 min at 4°C. The aqueous supernatant was transferred into a micro test tube, mixed with 0.6 ml isopropanol and centrifuged at 15,000 G for 15 min at 4°C. The precipitated total RNA was rinsed with 70% ethanol, air-dried, and then resuspended in RNase-free water. The concentration of the extracted total RNA was assessed using spectrophotometry. Next, the total RNA was treated with DNase Free™ reagent (Ambion, Austin, TX, USA) for 60 min, and then reverse-transcribed with Superscript II™ (Invitrogen, Carlsbad, CA, USA) at 37°C for 60 min using Random Primer™ (TaKaRa, Tokyo, Japan). The resultant cDNA mixture was stored in small aliquots at –20°C until further use. The integrity of each cDNA mixture was checked by amplification of glutaraldehyde 3-phosphate dehydrogenase (GAPDH) with ExTaq™ (TaKaRa), using the primer set 5'-ACCACAGTCCATGCCATCAC-3'/5'-TCCACCACCCTGTTGCTGTA-3'.

Complementary DNA Macroarray

Labeled cDNA probes were prepared with reagents provided with the Atlas Human Array kit (Clontech Laboratories, Palo Alto, CA, USA). For each specimen, 2.5μg of total RNA was incubated with Human Cytokine-Specific Primers (R&D Systems, Minneapolis, MN, USA) for 5 min at 65°C and then at 41°C. A mixture of reaction buffer, dNTP mix, 50 U of Moloney murine leukemia virus reverse transcriptase and [α-^{32}P] dATP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added to each sample, which was then incubated at 41°C for 60 min. The labeled cDNA probes were purified with column chromatography to remove unincorporated isotope (ProbeQuant™ G-50 Micro Columns; Amersham Pharmacia Biotech).

A nylon membrane containing bound cDNA clones corresponding to 375 different human genes (Human Cytokine Expression Array; R&D Systems) was prehybridized with a solution of hybridization buffer (ExpressHyb; Clontech) and salmon testes DNA at 68°C. Each labeled cDNA probe was mixed into prehybridization buffer and incubated overnight at 68°C with a membrane. After hybridization, the membrane was washed with wash solution 1 (2×standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)) and wash solution 2 (0.1×SSC, 0.1% SDS) at 68°C followed by a final wash of 2×SSC at room temperature. The washed membrane was wrapped in plastic wrap and exposed to a phosphor imaging screen. Imaging screens were scanned and analyzed with imaging software (Atlas Image software, Clontech). The signals on each array were corrected for background with an average for blank columns and standardized with a housekeeping gene present on the same membrane (glyceraldehyde phosphate dehydrogenase). The duplicated intensity signals for each gene were summed for data analysis. The ratio of gene expression levels was determined by dividing the signal intensity on the AAA array by that on the control array. Differential gene expression was considered significant when the signal ratio was greater than 5:1.

Primers and Probes for Real-Time RT-PCR

Using Primer Express software (Applied Biosystems, Foster, CA, USA), several sets of primers were designed for each of the genes. The primer set amplifying a target cDNA most effectively, which was evaluated by electrophoresis and staining with ethidium bromide, was selected for final use. Subsequently, the TaqMan probe inherent to each primer set was prepared, which was an oligonucleotide labeled with a reporter dye (FAM) at the 5'-end and a quencher dye (TAMRA) at the 3'-end.

Real-time RT-PCR was performed using an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems). The reaction solution was assembled in a volume of 25μl, which comprised TaqMan™ Universal PCR Master Mix (Applied Biosystems), forward and reverse primers (final concentration 300 nmol/L each), TaqMan probe (final concentration 200 nmol/L) and cDNA mixture (≈2.5 ng). The conditions for real-time RT-PCR were preheating at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of shuttle heating at 95°C for 15 s and at 60°C for 1 min. Throughout this study, the cDNA mixture from a particular sample was used to generate the working standard for quantitation of the cDNA of interest, which plots the relationship between the dilution of the standard cDNAs and the corresponding Ct value (the number of cycles necessary to obtain a threshold fluorescent signal). The initial quantity of the cDNA of interest in a certain cDNA mixture was calculated from the working standard and then normalized to that of GAPDH determined with TaqMan™ Assay Reagent Endogenous Control (Applied Biosystems). The normalized value for each target cDNA reflects the expression level of the corresponding gene in a test sample relative to the standard tissue.

Histology and Immunohistochemistry

Part of the plaque was placed in tissue fixative

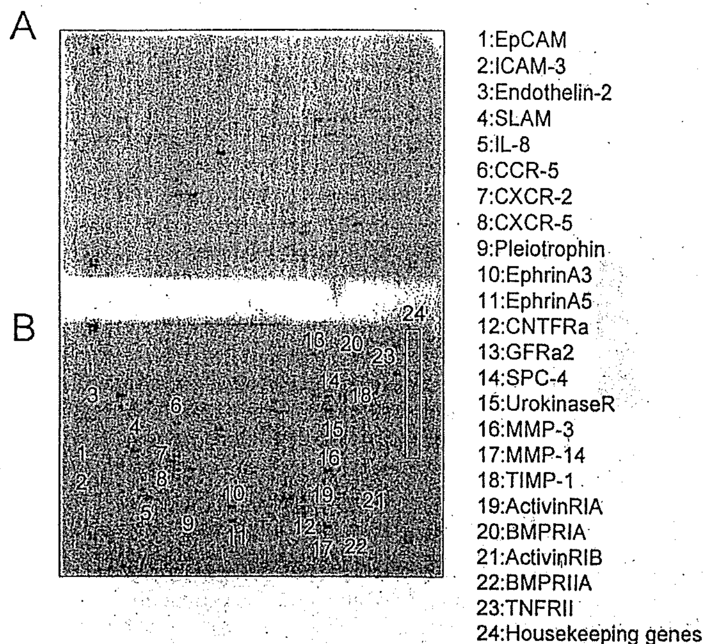

Fig 1. Representative result of the cDNA macroarray of the tissue from an abdominal aortic aneurysm. (A) Control, (B) diseased tissue.

Table 1 Sequence of Primer and Probe

IL8 sense	*TCTAGGACAAGAGCCAGGAAGAA*
IL8 antisense	*GGCCAGCTTGGAAGTCATGT*
IL8 TaqMan	*CACCGGAAGGAACCATCTCACTGTGTGTA*
CXCR2 sense	*TACATGGCTTGATCAGCAAGGA*
CXCR2 antisense	*GCCCTGAAGAAGAGCCAACA*
CXCR2 TaqMan	*TGCCCAAAGACAGCAGGCCTTCCT*
CCR2 sense	*GCCGCTGCTCATCATGGT*
CCR2 antisense	*TGCCTCTTCTTCTCGTTTCGA*
CCR2 TaqMan	*ACTCGGGAATCCTGAAAACCCTGCTTC*

The sequences of the primer and probe of MCP-1 were provided by on-line TaqMan gene expression assays (Assay ID Hs 00234140_m1).

(Histochoice, Hedwin, Baltimore, MD, USA). After overnight fixation, the samples were paraffin embedded and sectioned at 4μm intervals. Tissue sections were deparaffinized with xylene followed by immersion in graded alcohol. They were washed 3 times for 5 min each in phosphate-buffered saline (PBS) and blocked with bovine serum albumin for 60 min. Specimens were then incubated with primary antibodies (Fuji Chemical, Tokyo, Japan) overnight at 4°C. After they were washed in PBS, specimens were incubated with biotinylated rabbit anti-mouse IgG for 60 min at room temperature. Specimens were then washed with PBS, stained with horseradish peroxidase-conjugated streptavidin, and finally incubated with substrate solution for 1–15 min. The tissue sections were also stained with hematoxylin-eosin.

Statistical Analysis

The mean and standard error of triplicate data are presented. Statistical analysis was performed by Mann-Whitney test and Wilcoxon signed-rank test using Stat View 5.0 software (Abacus Concepts, Calabasus, CA, USA) on a Macintosh computer. A p-value <0.05 was considered significant.

Results

cDNA Macroarray for AAA Tissues

A representative autoradiograph of the human cytokine expression array after hybridization with cDNA probes derived from AAA and adjacent control tissues is shown in Fig 1. Although densitometric analysis revealed significant (>5-fold) upregulation of 97 of the 375 genes, 23 genes appeared to be overexpressed by visual inspection. Under these conditions, 10 cytokine-related genes were strongly overexpressed in comparison with those in the adjacent control tissues: Activin R1A (TGF b superfamily, 13:1), Activin R1B (TGF b superfamily, 12:1), BMP RIIA (TGF b superfamily, 12:1), CXCR-5 (chemokine receptor, 12:1), CXCR-2 (chemokine receptor, 11:1), IL-8 (chemokine, 9:1), CCR-6 (chemokine receptor, 9:1), BMP RIA (TGF b superfamily, 8:1), CXCR-1 (chemokine receptor, 7:1), and CXCR-6 (chemokine receptor, 7:1). It was interesting that IL-8 and its receptor, CXCR-2, were simultaneously upregulatated, suggesting their significant role in disease development. Therefore, these 2 genes were quantitatively determined by real-time RT-PCR with TaqMan probes (Table 1). Additionally, in some patients the expressions of MCP-1 and its receptor, CCR-2, were determined by the same procedures.

In AAA, the expression of IL-8 and CXCR-2 in CAS was 0.53±0.16 and 2.04±0.75, respectively and significantly greater than those in the adjacent control tissues in which the expressions were 0.11±0.04 and 0.29±0.10 ($p<0.01$), respectively. Under these conditions, the expressions of MCP-1 and CCR-2 in the diseased portion were slightly upregulated at 1.51±0.38 and 1.24±0.10, respectively, in comparison with those in adjacent control tissues (0.32±0.08 and 0.28±0.09, respectively, n=4).

In CAS, expression of IL-8 and CXCR-2 in CAS was 1.35±0.25 and 2.00±0.51, respectively and significantly greater than that in the adjacent control tissues where it was 0.60±0.16 and 0.58±0.21 ($p<0.05$), respectively (Fig 2). Under these conditions, there was no statistical significance

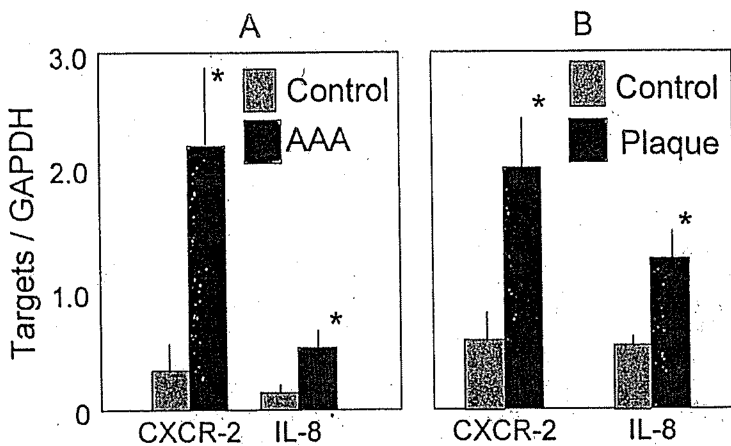

Fig 2. Expression of CXCR-2 and interleukin (IL)-8 in (A) abdominal aortic aneurysm (AAA) and (B) carotid artery stenosis (CAS).

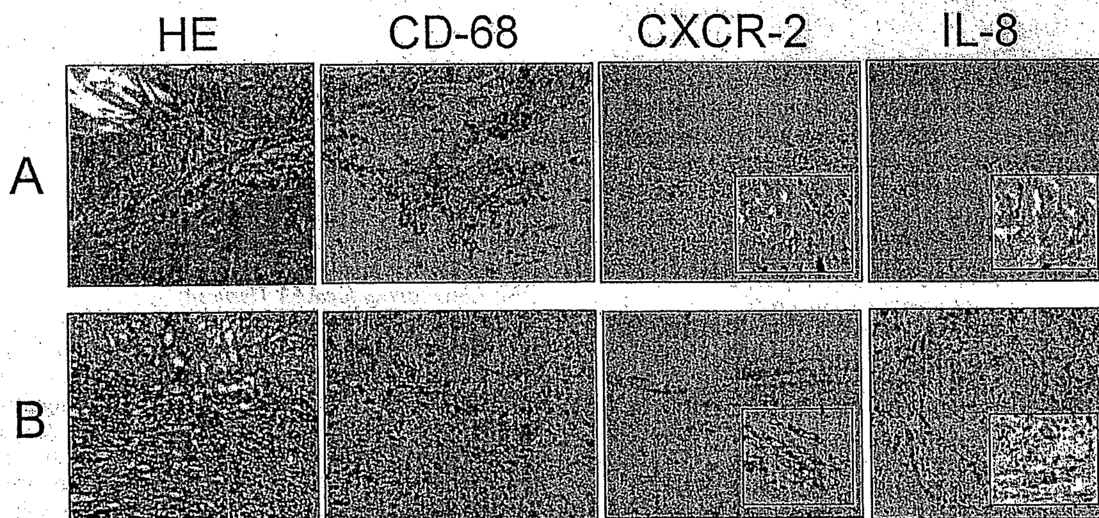

Fig 3. Immunohistochemisty of tissue samples from (A) abdominal aortic aneurysm and (B) carotid artery stenosis (Original magnification ×100; Insert: ×400).

in the expressions of MCP-1 and CCR-2 in diseased (0.44±0.15 and 2.72±1.76, respectively) and control (0.38±0.11 and 0.99±0.18, n=13) tissues.

Immunohistochemistry

In both AAA and CAS tissues, the control regions exhibited mild atherosclerosis in which a few CD-68 positive macrophages existed. The specimens of AAA consisted of thinned or thickened vascular tissue in which typical atheromatous plaques with infiltration of macrophages and lymphocytes were present. Plaque regions of CAS mainly consisted of a lipid-rich core and fibrous tissue in which CD-68 positive macrophages had accumulated, particularly in the shoulder regions of the atheroma. Under these conditions, IL-8 and CXCR-2 were mainly expressed in the macrophages, which were identified as CD68-positive cells in each tissue (Fig 3).

Discussion

In the present study, we used cDNA macroarray to try and determine the possible cytokine-related mediators that could contribute to the development of atherosclerosis, and we then quantitatively measured the expression of their mRNA using real-time RT-PCR. The inflammatory chemokine IL-8 and its receptor, CXCR-2, were demonstrated to be simultaneously upregulated in the diseased portions, suggesting their key role in the development of dilated and occlusive atherosclerotic diseases.

Determination Procedures for mRNA

The recent development of microchip- or membrane-based cDNA arrays has enabled examination of the simultaneous expression of multiple gene products of known identity, facilitating the identification of altered patterns of gene expression in a given tissue[9] Because this approach has the potential to reveal novel pathophysiologic insights, we first used it to characterize the simultaneous expression of approximately 375 gene products in human AAA, in which enough mRNA was collected to synthesize the cDNA, and to compare the expression profile of AAA with adjacent control tissues. We found that the expression of IL-8 (9-fold) and its receptor, CXCR-2 (11-fold) were simultaneously upregulated in the diseased portion.

However, it is necessary to reemphasize that the cDNA array is most valuable for detecting novel, unanticipated

alterations in gene expression, and that the strength of this method currently resides in its capacity to characterize patterns of gene expression rather than to quantify the expression of individual gene products. Thus, we performed quantitative analysis of gene expression with real-time RT-PCR, the accuracy of which to determine gene expression in human tissue has already been demonstrated elsewhere.[2]

Role of Inflammatory Chemokines in Dilated and Occlusive Atherosclerotic Diseases

IL-8, a C-X-C chemokine which was upregulated in both AAA and CAS, is produced by various types of cells on stimulation with inflammatory stimuli.[10–13] Low density lipoprotein (LDL)-deficient mice, irradiated and reconstituted with macrophages deficient in the murine IL-8 receptor homologue of human CXCR-2, show diminished macrophage recruitment to the lesion, suggesting a potential role for IL-8 in monocyte trafficking in vivo.[14] Moreover, IL-8 may contribute to the development of atherosclerosis by stimulating angiogenesis.[15] It exerts a variety of effects on leukocytes, particularly neutrophils,[16] and plays a critical role in the mobilization of stem cells through its induction of MMP-9.[17] In terms of MMP synthesis in dilated and occlusive atherosclerotic diseases, we previously reported significant upregulation of the expression of MMP-1 and -3 in AAA tissues, and MMP-1, -3 and -9 in CAS tissues.[2,3] MMP-9 is also upregulated in the infarct-related human coronary artery.[18,19] Therefore, upregulation of IL-8 might be related to overexpression of these MMPs in human atherosclerotic tissues.

IL-8 is also known to inhibit the production of the tissue inhibitor of matrix metalloproteinases (TIMPs), which are potent antagonists of MMPs in vessel tissue.[20] Our previous data indicate disproportional expression of MMPs/TIMPs in AAA as well as CAS.[2,3] Therefore, in the clinical setting upregulation of IL-8 could be related to this disproportional TIMPs expression in both AAA and CAS, thus enhancing the development of dilated or occlusive manifestation of atherosclerosis probably through the activation of proteinase activity. It has been suggested that the chemokine receptor CXCR-2 enhances monocyte recruitment and disease progression.[21] Indeed, the lack of CXCR-2 expression in bone marrow cells has been shown to be responsible for an almost 50% reduction in lesion development.[22]

There was no statistical significance in the expression of MCP-1 and CCR-2 in AAA and CAS tissues, which may be partly explained by the relatively high gene expression in the adjacent control tissues that already had minimal atherosclerotic changes. One might speculate that the MCP-1 and CCR-2 system, which is upregulated in the early stage of atherosclerosis, could be relatively downregulated in the established stage, such as the significant dilated and/or stenotic lesions studied by us. This might result in an altered balance in the expression of the contributing and suppression genes, which leads to the severity of the disease process.[1,4]

Clinical Implications and Study Limitations

Although the precise relationship between the inflammatory process of atherosclerosis and development of AAA and/or CAS, particularly in the established stage of the diseases, remains unclear, their frequent association and shared risk factors suggest common pathophysiologic mechanisms. This assertion is supported by the observation that both AAA and CAS exhibited increased expression of CXCR-2 as well as IL-8, both of which are thought to be important in inflammatory process of atherosclerosis, although we could not correlate the extent of disease severity with the levels of gene expression because of similar aneurysmal (>40 mm in diameter) and stenotic (>90%) lesions in the present cohort.

From the therapeutic point of view, it is interesting to note that the established anti-inflammatory pathways of HMG-CoA reductase inhibitors include the diminished expression of cytokines, such as IL-6 and IL-8, in cells implicated in atherogenesis or in human plasma.[23,24] In addition, oxydized LDL-cholesterol enhances the upregulation of the expression of CXCR-2 in monocytes, contributing to disease progression associated with dilation or occlusion.[25] Indeed, atorvastatin therapy, which suppresses the development of atherosclerosis as well as reduces the incidence of major adverse cardiac events,[26,27] could decrease the spontaneous release of IL-8 in mononuclear cells of patients with coronary artery disease.[28] Also, the effects of angiotensin-receptor inhibitors on suppression of atherosclerosis could be derived from inhibition of the overexpression of chemokines, such as IL-8, associated with reduction of macrophage accumulation in the lesion.[29] Although it would be indeed intriguing to examine whether the expressions of IL-8 and CXCR-2 in AAA and CAS could be affected by these "anti-inflammatory" drugs in the present cohort, we could not correlate gene expression level to treatment because of the variety of drugs and their doses. A prospective study of intensive use of HMG-CoA reductase inhibitors and/or angiotensin-receptor inhibitors for AAA and CAS patients may demonstrate the effectiveness of these agents on the expression of IL-8 and CXCR-2 and whether this is protective or not in the established stage of dilated and/or stenotic lesions.

One of the most important limitations in the present study is that we used the adjacent tissues, which were already involved in the disease, as the control. Therefore, the present study was not done with truly normal tissue. However, both control tissues did not show any dilated or occlusive lesions and might be considered to be in the "preaneurymal" or "preocclusive" state of the disease.

Even under these conditions, upregulation of IL-8 and associated CXCR-2 may have a crucial role in the development of manifested atherosclerotic disease.

Conclusions

We used a membrane-based cDNA macroarray and real-time RT-PCR to characterize the cytokine-related gene expression in AAA and CAS. Although the functional significance of the individual gene products that were altered in AAA and CAS will require further investigation, this study demonstrates the potential of cDNA expression array and real-time RT-PCR in elucidating the molecular mechanisms responsible for the development of AAA and CAS.

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Acute hyperglycemia is associated with adverse outcome after acute myocardial infarction in the coronary intervention era

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Purpose This study was undertaken to assess the association between acute hyperglycemia and inhospital outcome after acute myocardial infarction (AMI) in the percutaneous coronary intervention (PCI) era. We also assessed outcome of patients with a history of diabetes mellitus in the PCI era.

Methods Between January 2001 and December 2001, 1253 patients were admitted to the hospitals within 48 hours after the onset of AMI. Plasma glucose was measured at hospital admission. Acute hyperglycemia was defined as plasma glucose of >11 mmol/L (198 mg/dL), regardless of the diabetic status. Primary PCI was performed in 898 (72%) patients.

Results The inhospital mortality rate was significantly higher in patients with acute hyperglycemia than in patients without (16% vs 6%, $P < .001$). However, there was no significant difference in mortality between diabetic and nondiabetic patients (8% vs 9%, $P = .54$). Acute hyperglycemia was associated with a higher inhospital mortality rate both in nondiabetic patients (24% vs 6%, $P < .001$) and in diabetic patients (10% vs 5%, $P = .039$). Acute hyperglycemia was associated with a higher incidence of no reflow during PCI (21% vs 12%, $P < .001$), but diabetes was not (14% vs 15%, $P = .71$).

Conclusion Acute hyperglycemia, but not diabetes, was a predictor for inhospital mortality after AMI in the PCI era. No reflow occurred more frequently during PCI in patients with acute hyperglycemia, suggesting that microvascular dysfunction might have contributed to adverse outcome of these patients. (Am Heart J 2005;150:814-820.)

An increase of plasma glucose concentration is often observed during early hours after the onset of acute myocardial infarction (AMI) not only in patients with diabetes mellitus but also in patients without diabetes mellitus.[1] It has been reported that both acute hyperglycemia and diabetes mellitus are independently associated with adverse outcomes after AMI in the prereperfusion era and in the thrombolytic era.[2-7] Primary percutaneous coronary intervention (PCI) has been shown to be more effective than thrombolytic therapy for the treatment of AMI.[8] Recent progress in treatment of AMI might have changed the association between acute hyperglycemia and outcome after AMI. This study was undertaken to assess the association between acute hyperglycemia and inhospital outcome after AMI in the contemporary PCI era. In addition, because acute hyperglycemia was often confused with chronic hyperglycemia, the association between diabetes mellitus and outcome after AMI in the PCI era was also investigated.

Despite the recent progress in PCI, it has been shown that coronary stent has no benefit in terms of reducing no-reflow phenomenon.[9] No-reflow phenomenon is associated with adverse outcome after AMI.[10,11] It has been reported that hyperglycemia impairs microvascular function and may cause no-reflow phenomenon.[12]

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**See Appendices.*

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Table I. Baseline characteristics of patients with and those without acute hyperglycemia

	Acute hyperglycemia		
	Present (n = 378)	Absent (n = 875)	P value
Age (y)	70 ± 12	67 ± 12	.007
Men	236 (62%)	648 (74%)	<.001
Hypertension	209 (55%)	489 (56%)	.85
Previous angina	115 (30%)	327 (37%)	.02
Previous infarction	59 (16%)	107 (12%)	.11
Diabetes mellitus	231 (61%)	166 (19%)	<.001
Time to admission (h)	5.7 ± 8.0	7.1 ± 9.3	.01
Killip classes 2 to 4	133 (35%)	143 (16%)	<.001
ST elevation	308 (81%)	745 (85%)	.11
Medication before infarction			
Aspirin	43 (11%)	88 (10%)	.49
ACE inhibitor	25 (7%)	54 (6%)	.77
ARB	10 (3%)	21 (2%)	.80
β-Blocker	18 (5%)	45 (5%)	.78
Ca channel blocker	89 (24%)	219 (25%)	.57
Nicorandil	13 (3%)	33 (4%)	.77
Statin	32 (8%)	54 (6%)	.15
Any of above medications	132 (35%)	283 (32%)	.37
Oral hypoglycemic drug	114 (30%)	58 (7%)	<.001
Insulin	49 (13%)	17 (2%)	<.001
Reperfusion therapy			
Thrombolysis	40 (11%)	64 (7%)	.06
Balloon angioplasty	43 (11%)	130 (15%)	.10
Stent	219 (58%)	506 (58%)	.97
Bypass surgery	9 (2%)	9 (1%)	.08
Neither	67 (18%)	168 (19%)	.54

Acute hyperglycemia was defined as plasma glucose of >11 mmol/L at admission, regardless of the diabetic status. *ACE*, Angiotensin-converting enzyme; *ARB*, angiotensin receptor blocker.

Table II. Baseline characteristics of patients with and those without diabetes mellitus

	Diabetes mellitus		
	Present (n = 397)	Absent (n = 856)	P value
Age (y)	67 ± 11	69 ± 13	.007
Men	273 (69%)	611 (71%)	.35
Hypertension	255 (64%)	443 (52%)	<.001
Previous angina	128 (32%)	314 (37%)	.12
Previous infarction	70 (18%)	96 (11%)	.002
Plasma glucose at admission (mmol/L)	13.2 ± 5.6	8.7 ± 3.4	<.001
Time to admission (h)	6.5 ± 8.9	6.8 ± 9.0	.70
Killip classes 2 to 4	102 (26%)	174 (20%)	.03
ST elevation	322 (81%)	731 (85%)	.06
Medication before infarction			
Aspirin	60 (15%)	71 (8%)	<.001
ACE inhibitor	37 (9%)	42 (5%)	.004
ARB	14 (4%)	17 (2%)	.11
β-Blocker	22 (6%)	41 (5%)	.57
Calcium-channel blocker	112 (28%)	196 (23%)	.04
Nicorandil	21 (5%)	25 (3%)	.04
Statin	45 (11%)	41 (5%)	<.001
Any of above medications	157 (40%)	258 (30%)	.001
Oral hypoglycemic drug	172 (43%)	0 (0%)	<.001
Insulin	66 (17%)	0 (0%)	<.001
Reperfusion therapy			
Thrombolysis	116 (29%)	216 (25%)	.14
Balloon angioplasty	59 (15%)	114 (13%)	.46
Stent	206 (52%)	519 (61%)	.004
Bypass surgery	9 (2%)	9 (1%)	.10
Neither	77 (19%)	158 (18%)	.69

Diabetes mellitus was defined as previous or current diagnosis of diabetes mellitus, regardless of the glycemic status at admission.

A second objective of this study was to ascertain whether acute hyperglycemia was associated with no-reflow phenomenon during PCI for AMI.

Methods

Patients

The JACSS is a retrospective observational multicenter study conducted at 35 medical institutions.[13] Between January 2001 and December 2001, 1640 consecutive patients who were admitted to the participating institutions within 48 hours after the onset of AMI were enrolled in the JACSS. Plasma glucose was measured at the time of hospital admission in 1253 (76%) patients, who constituted the current study group. Acute myocardial infarction was defined by a combination of 2 of the following 3 characteristics: chest pain consistent with ongoing myocardial ischemia persisting longer than 30 minutes, ischemic electrocardiographic changes, and peak creatine kinase value more than twice the normal upper limit.

Acute hyperglycemia was defined as plasma glucose of >11 mmol/L (198 mg/dL) at admission, regardless of the diabetic status. Patients were thought to have diabetes mellitus if they had previous or current diagnosis of diabetes mellitus, regardless of the glycemic status at admission. The study protocol was reviewed and approved by the ethical committee of each participating institution.

Coronary angiography and PCI

Percutaneous coronary intervention was performed as reperfusion therapy in 898 (72%) patients: coronary stent in 725 (58%) patients and conventional balloon angioplasty in 173 (14%) patients. The allocation of coronary angiography and reperfusion therapy was determined by physician's decision. The perfusion status of the infarct artery was assessed in accordance with the TIMI study classification.[14] Angiographic no-reflow was thought to be present if the perfusion of the infarct artery was TIMI-0 to TIMI-2 flow during PCI, despite the absence of stenosis of >50%, flow-limiting coronary dissection, or hypotension. Treatment of no-reflow, including intracoronary infusion of vasodilators, depended on the physician's decision.[15] Final TIMI flow grade was assessed on the final shot of the acute angiography.

End points

The primary end point was all-cause inhospital mortality. Other important clinical outcomes, including cardiac death, reinfarction, unstable angina, heart failure, and stroke, were also assessed during hospitalization. In patients who under-

Table III. The incidence of inhospital mortality and MACE

	Acute hyperglycemia			Diabetes mellitus		
	Present (n = 378)	Absent (n = 875)	*P* value	Present (n = 397)	Absent (n = 856)	*P* value
Death	60 (16%)	50 (6%)	<.001	32 (8%)	78 (9%)	.54 (ns)
MACE	76 (20%)	84 (10%)	<.001	56 (14%)	104 (12%)	.34 (ns)

MACE, Major adverse cardiac events including cardiac death, reinfarction, unstable angina, heart failure, and stroke; *ns*, not significant.

went PCI as reperfusion therapy, appearance of angiographic no-reflow during PCI was reported.

Data analysis

Statistical analysis was performed with the χ^2 test for categorical variables. The *t* test and analysis of variance were used for continuous variables. To assess the relationship between plasma glucose level and mortality, Cox proportional hazards regression model was used, and odds ratio (OR) and 95% CI were obtained. In this analysis, plasma glucose was used as a continuous variable. Multivariate analysis was performed adjusting diabetes mellitus, age, sex, hypertension, previous angina, previous infarction, time to admission, Killip class, ST elevation, use of cardiovascular medication before AMI, and PCI as reperfusion therapy. Differences were considered significant if the *P* value was <.05.

Results

Patient characteristics

Acute hyperglycemia was associated with older age, more women, more diabetes mellitus, more Killip class ≥2, less previous angina, and shorter time from the onset of AMI to admission (Table I). Diabetes mellitus was associated with younger age, more hypertension, more Killip class ≥2, more previous infarction, higher plasma glucose on admission, and less stent implantation (Table II).

There was no significant difference in medications before AMI between patients with acute hyperglycemia and patients without, except for more use of oral hypoglycemic drugs and insulin in patients with acute hyperglycemia. The use of cardiovascular medications was significantly more frequent in diabetic patients than in nondiabetic patients.

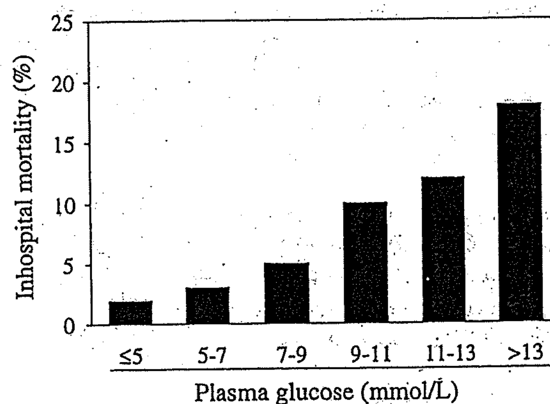
Hemoglobin A1c was measured during hospitalization in 561 (45%) patients. Hemoglobin A1c was 5.4% ± 0.5% in nondiabetic patients without acute hyperglycemia, 5.7% ± 0.8% in nondiabetic patients with acute hyperglycemia, 6.4% ± 1.1% in diabetic patients without acute hyperglycemia, and 8.0% ± 1.7% in diabetic patients with acute hyperglycemia ($P < .001$).

Inhospital outcomes

The inhospital mortality rate was significantly higher in patients with acute hyperglycemia than in patients without (Table III). Major adverse cardiovascular events, including cardiac death, reinfarction, unstable angina, heart failure and stroke, occurred more frequently in patients with acute hyperglycemia. The inhospital mortality increased as plasma glucose increased (Figure 1). An increase of 1 mmol/L (18 mg/dL) in plasma glucose was associated with an increase in mortality risk of 12% in univariate analysis (OR 1.12, 95% CI [1.08-1.16], $P < .001$) and 10% in multivariate analysis (OR 1.10, 95% CI [1.05-1.15], $P < .001$). On the contrary, there was no significant difference in the inhospital mortality rate and the major adverse cardiovascular events rate between diabetic and nondiabetic patients. Inhospital mortality of patients with acute hyperglycemia was twice as high as mortality of patients with diabetes mellitus (16% vs 8%). Acute hyperglycemia was associated with a higher inhospital mortality rate both in nondiabetic patients (24% vs 6%, $P < .001$) and in diabetic patients (10% vs 5%, $P = .039$).

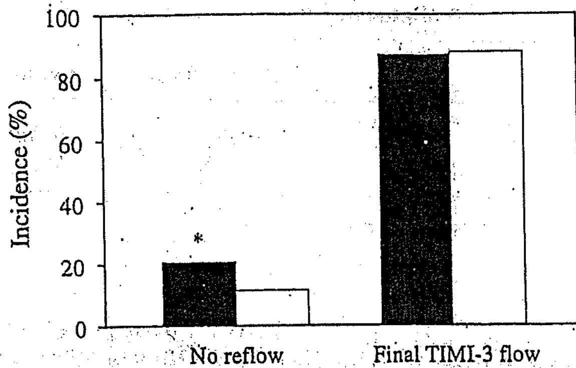
Peak creatine kinase was obtained in 1187 (94%) patients. Peak creatine kinase was significantly higher in

Figure 1



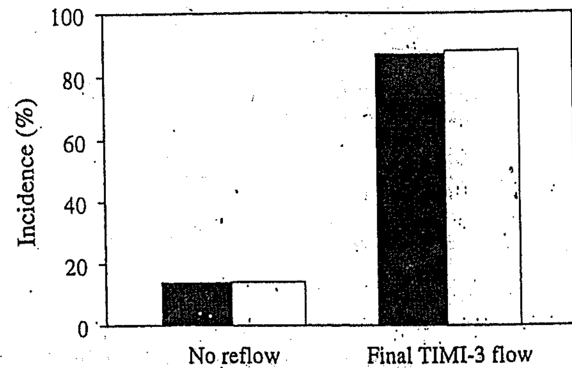
The inhospital mortality rate increased as plasma glucose increased (2% in patients with plasma glucose ≤5 mmol/L, 3% in patients with plasma glucose 5 to 7 mmol/L, 5% in patients with plasma glucose 7 to 9 mmol/L, 10% in patients with plasma glucose 9 to 11 mmol/L, 12% in patients with plasma glucose 11 to 13 mmol/L, and 18% in patients with plasma glucose >13 mmol/L; $P < .001$).

Figure 2



During coronary intervention, the incidence of angiographic no-reflow was more frequent in patients with acute hyperglycemia (black bars) than in patients without acute hyperglycemia (white bars). However, the incidence of final TIMI-3 was not different, *P < .001.

Figure 3



There was no significant difference in the incidence of angiographic no-reflow and final TIMI-3 flow between patients with diabetes mellitus (black bars) and patients without diabetes mellitus (white bars).

patients with acute hyperglycemia than in patients without (3176 ± 2945 vs 2698 ± 2557 IU/L, P = .005). However, there was no significant difference in peak creatine kinase between diabetic and nondiabetic patients (2695 ± 2580 vs 2905 ± 2730 IU/L, P = .21).

Angiographic no-reflow during PCI

See Figures 2 and 3. Among 898 patients who underwent PCI, angiographic no-reflow occurred in 128 (14%) patients during the procedure. Angiographic no-reflow was associated with a higher inhospital mortality rate (12% vs 5%, P = .01), a higher major adverse cardiovascular events rate (18% vs 9%, P = .003), and higher peak creatine kinase (4094 ± 3575 vs 2915 ± 2569 IU/L, P < .001). The incidence of angiographic no-reflow was significantly higher in patients with acute hyperglycemia than in patients without (21% vs 12%, P < .001) but was not different between diabetic and nondiabetic patients (14% vs 15%, P = .71). Acute hyperglycemia was associated with angiographic no-reflow both in nondiabetic patients (26% vs 12%, P < .001) and in diabetic patients (17% vs 9%, P = .036). The incidence of final TIMI-3 flow was not different regardless of the presence or absence of acute hyperglycemia (87% vs 88%, P = .84) or diabetes mellitus (87% vs 88%, P = .73).

Discussion

Although it has been demonstrated that increased plasma glucose at admission is associated with adverse outcome after AMI in the reperfusion era, most of these study patients were treated with thrombolysis, and there were few data on patients undergoing primary PCI.[6,7,16,17] Recently, Wahab et al[7] have reported that plasma glucose is an independent predictor of mortality after AMI in the thrombolytic era. However, only 34% of the study patients underwent thrombolytic therapy, and PCI was performed in <10% of the patients. JACSS is a contemporary multicenter study in which >70% of the patients underwent PCI as reperfusion therapy. We used the same definition of acute hyperglycemia as did Wahab et al, so that our result could be compared with previous findings. This study showed that acute hyperglycemia was associated with adverse inhospital outcome after AMI in the contemporary PCI era.

It remains controversial whether acute hyperglycemia predisposes to adverse outcome or is simply a consequence of large infarct size. A higher incidence of Killip class ≥2 suggests that acute hyperglycemia may reflect extensive myocardial damage. However, recent experimental studies have suggested that hyperglycemia per se exacerbates myocardial damage in AMI. Hyperglycemia increases interstitial fibrosis and myocyte apoptosis that exaggerate left ventricular remodeling.[18] Also, hyperglycemia abolishes the cardioprotective effect of ischemic preconditioning by closing K_{ATP} channels.[19,20] A recent clinical study reported that acute hyperglycemia was associated with impaired predischarge left ventricular ejection fraction in patients with AMI, independently of acute left ventricular ejection fraction.[21]

Another potential mechanism for the association between acute hyperglycemia and adverse outcome is microvascular dysfunction. Experimental studies have reported that hyperglycemia aggravates platelet-dependent thrombosis, increases circulating adhesion molecules that augment capillary leukocyte plugging, attenuates endothelium-dependent vasodilation, and reduces collateral blood flow by adversely affecting nitric oxide availability.[22-25] These changes impair microvascular function. Recently, Iwakura et al[12]

reported that hyperglycemia was associated with no-reflow phenomenon on myocardial contrast echocardiography in patients with angiographically successful reperfusion after PCI. No-reflow phenomenon is a strong predictor for adverse outcome after AMI.[10,11] We also showed that angiographic no-reflow occurred more frequently in patients with acute hyperglycemia, suggesting that impaired microvascular function might have contributed to adverse outcome after AMI in patients with acute hyperglycemia.

Interestingly, there was no significant difference in inhospital outcome between diabetic and nondiabetic patients. Previous studies have demonstrated that diabetes mellitus is associated with adverse outcome after thrombolysis for AMI.[26,27] In several studies that used noninvasive indices, reperfusion was achieved less frequently after thrombolysis in patients with diabetes mellitus than in patients without.[28] Angeja et al[29] reported that diabetes mellitus was associated with less complete ST-segment resolution after thrombolysis, even in patients with TIMI-3 flow. However, the incidence of complete ST-segment resolution was similar between diabetic and nondiabetic patients after PCI. More effective reperfusion by PCI, as compared with thrombolysis, may improve outcome of diabetic patients. Recent studies have reported that diabetes mellitus did not increase short-term mortality after AMI,[30-32] especially in non-insulin-requiring patients with diabetes.[33] Lower incidence of non-insulin-requiring patients with diabetes in this study (83% of diabetic patients) may also account for relatively favorable outcome of patients with diabetes mellitus. In addition, the use of cardiovascular medications before admission for the index episode of AMI was more frequent in diabetic patients than in nondiabetic patients. Pharmacological cardiovascular prevention might have offset the adverse effect of diabetes mellitus on short-term outcome after AMI.[30]

In this study, mortality was higher in nondiabetic patients with acute hyperglycemia than in diabetics with acute hyperglycemia. Although experimental studies have reported that diabetic hearts are tolerant to ischemia in some conditions,[33] it is unclear whether it may occur in human beings. One possible interpretation of this data is that unrecognized diabetes mellitus is a marker of adverse outcome in patients with AMI. However, the mean value of hemoglobin A1c of nondiabetic patients with acute hyperglycemia was significantly lower than that of diabetic patients with acute hyperglycemia ($P < .001$). Recent studies have reported that plasma glucose at admission is associated with increased mortality even after adjustment of hemoglobin A1c.[34] It is thus unlikely that the adverse outcome of nondiabetic patients with acute hyperglycemia is as a result of chronic hyperglycemia of undiagnosed diabetes mellitus.

This is a retrospective and observational study. However, it included all consecutive patients who were admitted to the participating institutions during the first year of the new millennium. Patients received contemporary management and >70% of the patients underwent PCI. Plasma glucose at admission was reported only in 76% of the patients enrolled in the JACSS. However, there was no significant difference in the incidence of diabetes mellitus (32% vs 28%, $P = .31$) and the inhospital mortality rate (9% vs 11%, $P = .34$) between patients with measurement of plasma glucose at admission and patients without. Because diabetes mellitus was defined as previous or current diagnosis of diabetes mellitus at the time of hospital admission, some of diabetic patients may not have been diagnosed as such. Oral glucose tolerance test was not usually performed during hospitalization, and it was not assessed how often nondiabetic patients with acute hyperglycemia at admission had newly diagnosed diabetes mellitus by the time of hospital discharge. We did not assess microvascular flow by using myocardial blush grade or TIMI frame count, which might have provided additional information.

In conclusion, acute hyperglycemia, but not diabetes mellitus, was associated with inhospital mortality after AMI in the PCI era. Angiographic no-reflow occurred more frequently during PCI in patients with acute hyperglycemia, suggesting that microvascular dysfunction might have contributed to adverse outcome of these patients.

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Appendix A

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Appendix B

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Successful Excision of a Cystic Tumor of the Atrioventricular Nodal Region

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Cystic tumor of the atrioventricular nodal region is a rare cardiac primary tumor that can cause heart blockage and sudden death. Antemortem diagnosis and successful excision of the atrioventricular nodal region are extremely rare. A 45-year-old woman who presented with palpitations is reported. Electrocardiography revealed first-degree atrioventricular block. Echocardiography, computed tomography, and magnetic resonance imaging scans revealed a cystic mass attached to the interatrial septum. Complete surgical excision of the mass was achieved, although placement of a permanent pacemaker was required for complete heart blockage. Histopathological examination revealed the mass to be a cystic tumor of the atrioventricular nodal region. A 5-year follow-up has revealed no sign of recurrence. (*Circ J* 2005; **69:** 1293 – 1294)

Key Words: Atrioventricular node; Cystic tumor; Heart block; Pacemaker

Cystic tumor of the atrioventricular nodal region is a rare primary cardiac tumor. It can cause various degrees of heart blockage, and is the smallest tumor capable of causing sudden death.[1–3] Although there have been approximately 70 reported cases of atrioventricular nodal region in published reports to date, most were diagnosed postmortem. Antemortem diagnosis and successful excision of this type of tumor are extremely rare. Here we report a case of cystic tumor of the atrioventricular nodal region in which the tumor was detected preoperatively and successfully excised.

Case Report

A 45-year-old woman who presented with dyspnea on effort and palpitations visited her primary care physician. A resting electrcardiogram (ECG) revealed sinus rhythm and first-degree atrioventricular block. Sporadic ventricular paroxysmal contraction was found on Holter ECG. Because echocardiography showed an intracardiac tumor, she was referred to our institute for surgical evaluation. Transesophageal echocardiography and computed tomography (Fig 1A) revealed a 28–27 mm circular tumor with no stalk on the interatrial septum (IAS).[4] On magnetic resonance imaging (MRI), the tumor was of high intensity on T1-weighted images and isointense with myocardium on T2-weighted images (Fig 1B).

She underwent surgery to resect the tumor. Under a median sternotomy and standard cardiopulmonary bypass with warm blood cardioplegia, the right atrium was opened, to reveal a 30 mm round cyst attached to the IAS in the area of the triangle of Koch. The cyst was incised and yellow caseous material was found within it. Rapid cytodiagnosis was done and revealed neither malignant cells nor bacteria in the fluid. The IAS at the portion of the

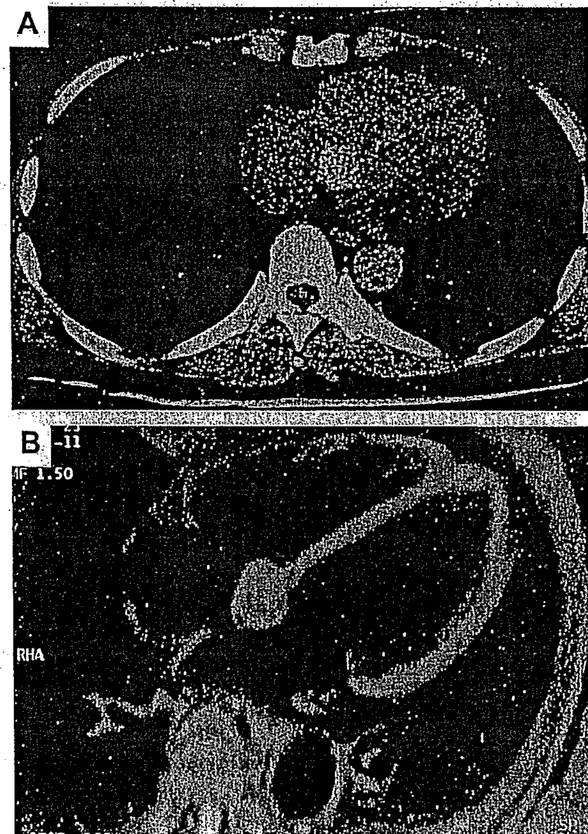

Fig 1. (A) Computed tomography scan and (B) magnetic resonance imaging showing intracardiac tumor with a broad connection to the interatrial septum.

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