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Expression of Interleukin-1β, Interleukin-1 Receptor, and Interleukin-1 Receptor Antagonist mRNA in Rat Carotid Artery after Balloon Angioplasty

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Interleukin-1β (IL-1β) is a pleiotropic cytokine capable of inducing smooth muscle activation and leukocyte recruitment in restenosis and atherosclerosis. Our present study investigated the expression of IL-1β, IL-1 receptor antagonist (IL-1ra), and IL-1 receptor (IL-1RI and IL-1RII) mRNA in carotid artery after balloon angioplasty using semiquantitative reverse transcription/polymerase chain reaction (RT/PCR) and Northern analysis. Time course studies revealed that IL-1β mRNA was rapidly induced at 6 h (30-fold increase over control, $P < 0.001$) post balloon injury and diminished to basal levels at 24 h. The increased expression of IL-1ra mRNA was delayed, reaching a peak at 24 h (400-fold increase, $P < 0.001$) and sustained up to 14 days. The expression of IL-1RII mRNA was remarkably similar to that of IL-1β, whereas the IL-1RI (the signaling receptor) mRNA expression was delayed (significantly induced at day 1; 14.2-fold increase, $P < 0.01$) post balloon injury. Immunohistochemical studies revealed a strong induction of IL-1β in the area with actively proliferating and migrating smooth muscle cells (i.e., in the inner medial layer at day 1 and in neointima at day 14 after balloon injury). The differential but concomitant expression of IL-1β, IL-1ra, IL-1RI, and IL-1RII mRNAs after balloon angioplasty suggests that each of these IL-1 system components may play a distinct role in neointima formation.

Key Words: **angioplasty; cytokines; experimental; gene expression; interleukin-1; restenosis; smooth muscle; vasculature.**

Interleukin-1β (IL-1β) is a proinflammatory cytokine and a potent inducer for a number of genes including other cytokines and growth factors, metalloproteinases and prostaglandins (1–3). IL-1β is also a chemoattractant and mitogen for smooth muscle cells (SMCs) (1–3). IL-1β can be synthesized and released by inflammatory cells, endothelial cells and SMCs in vascular disease tissues (1–4). It is suggested that local production of IL-1β may alter many important biological functions of vascular cells, including stimulating SMC proliferation (1) and inducing adhesion molecule expression in endothelial cells and thus facilitating leukocyte recruitment during inflammation and atherogenesis (5–7).

Neointimal formation is a pathophysiological condition characterized by SMC activation, migration and proliferation. A number of genes including growth factors (e.g., PDGF and bFGF) and cytokines (e.g., TNF-α and MCP-1) have been identified and postulated for their roles in neointimal formation. Since IL-1β has been recognized as one of the most potent mediators responding to vascular injury, its expression and activity in neointimal formation is of great interest. IL-1 signal transduction is mediated though a specific cell surface receptor, an 80 kDa glycoprotein known as type I IL-1 receptor (IL-1RI) (3, 8). The IL-1 signal transduction can be modulated by an endogenous interleukin-1 receptor antagonist (IL-1ra; a 23–25 kDa glycosylated protein) and a C-terminal truncated interleukin-1 receptor type II (IL-1RII; a 68 kDa glycoprotein) (3, 8). The difference in the cytoplasmic sequences of the two receptors allows IL-1RI but not IL-1RII to engage in intracellular signal transduction (3, 9, 10), while IL-1RII only acts as a binding "sink" or reservoir for IL-1β. IL-1ra has a higher binding affinity for IL-1RI than IL-1α and IL-1β and therefore can block IL-1 activity *in vitro* and *in vivo* systems.

Since the biological action of IL-1β in neointimal formation involves each IL-1 component, in the present study we evaluated the temporal expression profile of IL-1β, IL-1ra and two IL-1 receptors thoughout the

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initiation, evolution and establishment of neointimal formation in rat carotid artery subjected to balloon angioplasty.

MATERIALS AND METHODS

Left common carotid artery balloon angioplasty. Left common carotid artery balloon angioplasty was performed on male Sprague-Dawley rats (400 g; Charles River, Wilmington, MA) under sodium pentobarbital anesthesia (65 mg kg^{-1}, i.p.) as described previously (11). Briefly, following an anterior midline incision, the left external carotid artery was identified and cleared of adherent tissue, allowing the insertion of a 2-F Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Santa Ana, CA). The catheter was guided at a fixed distance (5 cm) down the common carotid arteries to a point such that the tip of the catheter was proximal to the aortic arch where the balloon was inflated and withdrawn back to its point of insertion. This procedure was performed a total of three times after which the catheter was removed and a suture was tied around the external carotid artery to prevent exanguination. Finally the wound was closed using 9 mm Autoclips (Clay Adams, Parsippany, NJ). Thoughout the surgical procedure, body temperature was maintained at 37 ± 1°C using a K-20-F water blanket (American Hamilton, Cincinnati, OH).

Animals were allowed to recover from surgery and were housed in pairs in Plexiglass cages on 12 h light/dark cycle with access to standard laboratory chow and drinking water *ad libitum*. All surgical interventions were performed in accordance with the guidelines of the Animal Care and Use Committee, SmithKline Beecham and the American Association for Laboratory Animal Care.

Left common carotid arteries were isolated from rats immediately following exanguination under sodium pentobarbital anesthesia (65 mg kg^{-1}, i.p.). Vessels that included the areas devoid of endothelium and containing endothelium were removed at the following time points: 0 (control), 6 h, 1, 3, 7, and 14 days after surgery. Once isolated, vessels were immediately frozen in liquid N_2 and stored at −70°C for RNA preparation. Each of the six individual time points consisted of vessels pooled from 3 rats and 5 separate pooled samples were analyzed in the present studies.

Reverse transcription and polymerase chain reaction. Carotid arteries were homogenized in an acid guanidinium thiocyanate solution and extracted with phenol and chloroform as described previously (12, 13). For reverse transcription, total cellular RNA (3 μg/each sample) isolated from 0, 6 h, 1, 3, 7, and 14 days following carotid artery balloon angioplasty was reverse transcribed in the presence of 200 U of RNase H^- SuperScript II reverse transcriptase (Gibco BRL) and 1 μg of oligo$(dT)_{12-18}$ primer at 37°C for 60 min according to manufacturer's specification. The resultant cDNA products were phenol-chloroform extracted and ethanol precipitated. The cDNA pellets were then dried under speed vacuum, resuspended in 120 μl of TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) and stored at −20°C until required for PCR amplification.

The semiquantitative PCR was carried out similar to that described in detail previously (14). A reference gene (rpL32 in this case) that has been shown previously for its constant expression after balloon angioplasty (12) was used as an internal control for the co-amplification with the genes to be examined. PCR primers used for amplification of IL-1β, IL-1ra, IL-1RI, IL-1RII and rpL32 were synthesized according to published sequences (Table 1). The optimal amplification conditions for the RT templates, amplification cycles and the ^{32}P-labeled primers were determined as described in detail previously (18). Based upon these initial results, the linear portion of the amplification was determined for both the tested genes and the internal standard (i.e., rpL32 in the present study). Therefore, the following conditions were chosen as a standard for PCR reactions in a total 50 μl of reaction mixture: RT products from 0.1 μg RNA, 28 cycles for IL-1β and IL-1ra or 30 cycles for IL-1RI and IL-1RII. The reaction mixture contained 1×10^6 cpm ^{32}P-labeled reverse primers for the examined genes and 5×10^4 (for 28 cycles) or 4×10^4 cpm (for 30 cycles) for rpL32, together with 100 ng of each non-radioactive forward and reverse primers (Table 1). The amplification was carried out using 2.5 units of TaqAmpli polymerase (Perkin-Elmer Cetus) in a thermocycler (Perkin-Elmer Cetus) according to the conditions described previously (14): initial denaturation, 3 min at 94°C; initial annealing, 1 min at 54°C; initial extension, 3 min at 72°C. The subsequent cycles were: denaturation, 15 s at 94°C; annealing, 20 s at 54°C; extension, 1 min at 72°C. Ten μl of the PCR product was electrophoresed though a 6% polyacrylamide gel. The gel was dried and subjected to autoradiography at room temperature. The signal intensity was quantitated using PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis, and the relative mRNA levels were determined by calculating the ratio of IL-1β, IL-1ra, IL-1RI or IL-1RII to rpL32 in each co-amplified sample.

TABLE 1

Oligonucleotide Primers of IL-1β, IL-1ra, IL-1RI, IL-1RII, and rpL32 Used for PCR[a]

Gene	Sequence	F/R	Position
IL-1β	5′-CCTGTCCTGTGTGATGAAAG-3′	F	628–647
	5′-CCCATACACACGGACAACTA-3′	R	1161–1180
IL-1ra	5′-TCTGCAGGGGACCTTACAGT-3′	F	8–27
	5′-GGTCTTCCTGGAAGTAGAAC-3′	R	513–532
IL-1RI	5′-AGATGGAAGGACCTATGATG-3′	F	1512–1531
	5′-TGCAGCATCTGACGACAGGA-3′	R	2133–2152
IL-1RII	5′-GGCAAGGAATACAACATCAC-3′	F	802–821
	5′-TGGTTGTCAGTCGGTAGCTT-3′	R	1312–1331
rpL32	5′-GTGAAGCCCAAGATCGTC-3′	F	28–45
	5′-GAACACAAAACAGGCACAC-3′	R	422–440

[a] Base pair positions are those given in the published cDNA sequences for rat IL-1β (GenBank M98820), IL-1ra [15], IL-1RI [16], IL-1RII [17], and rpL32 [18]. The letters F and R represent forward and reverse oligonucleotides, respectively.

Northern blot analysis. Total RNA samples (10 μg/lane) extracted from pooled (n = 10) rat carotid artery after balloon angioplasty were resolved by electrophoresis, transferred onto a GeneScreen Plus membrane (DuPont-New England Nuclear, Boston, MA) and subjected to Northern hybridization as described in detail previously (12). Rat IL-1β, IL-1ra and ribosomal protein L32 (rpL32) cDNA fragments were generated by RT-PCR and isolated by electrophoresis. The cDNA was uniformly labeled with [α-^{32}P]dATP using a random-priming DNA labeling kit. Hybridization of each probe was carried out overnight with 1×10^6 cpm/ml of probe at 42°C in 5 × SSPE (750 mM NaCl, 50 mM NaH_2PO_4, pH 7.6, 5 mM EDTA), 50% formamide, 5 × Denhardt's solution, 2% SDS, 100 μg/ml polyA and 200 μg/ml boiled salmon sperm DNA. The membranes were washed in 2 × SSPE, 2% SDS at 65°C for 1–2 h with a change every 30 min, then autoradiographed at −70°C with a Cronex Lightning-Plus intensifying screen for various times depending upon the signal intensity. A probe was stripped from the membranes in 10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 1% SDS for 20 min at 95°C and then washed in 2 × SSPE for 10 min prior to re-hybridization with the other probe.

Immunohistochemistry. Left common carotid arteries that had undergone balloon angioplasty were removed from rats after *in situ* perfusion fixation (90 mm Hg) using 10% (wt/vol) phosphate-buffered formalin (Baxter Scientific Products). Following standard histological processing and embedding in paraffin, 6 μm-thick sections were prepared for immunoperoxidase staining using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Briefly, endogenous peroxidase was quenched

with 0.3% H_2O_2 in methanol for 30 min. Nonspecific immunoglobulin binding sites were blocked with normal goat serum for 1 h and then the sections were incubated with primary antibody (rabbit anti-rat IL-1β, 10 μg/ml, Endogen, Cambridge, MA) for 1 h at room temperature. As a control, serial sections were treated with rabbit anti-rat IL-1β antibody that had been preincubated with recombinant IL-1β (1 μM, Gibco BRL) for 30 min at room temperature. The sections were then incubated for 30 min with a biotinylated goat anti-rabbit IgG secondary antibody (1:200, Vector Laboratories) followed by 30 min of incubation with the Vectastain ABC reagent solution. Immunoglobulin complexes were visualized upon incubation with 3,3′-diaminobenzidine (DAB, Vector Laboratories) at 0.5 mg/ml in 50 mM Tris-HCl, pH 7.4 and 3% H_2O_2. DAB staining was enhanced by treating the sections for 10 s with DAB Enhancing Solution (Vector Laboratories). Sections were washed, counterstained with Gill's Hematoxylin, cleared, mounted with Aquamount (Polysciences, Warrington, PA), and then examined by light microscopy.

Statistical analysis. Statistical evaluation was performed using one-way ANOVA followed by a Fisher's protected *t* test. The results are expressed as mean ± standard error. Significance was accepted for $p < 0.05$ by comparing the relative mRNA levels with the controls (time = 0).

RESULTS

Temporal expression of IL-1β, IL-1ra, IL-1RI, and IL-1RII mRNA in rat carotid artery after balloon angioplasty. Representative autoradiographs for IL-1β and IL-1ra (Fig. 1A) and for IL-1RI and IL-1RII (Fig. 1B) mRNA expression by means of semi-quantitative RT/PCR are illustrated. The quantitative data for the mRNA expression of IL-1β, IL-1ra and the two IL-1 receptors (n = 5), after normalizing to a co-amplified house-keeping gene rpL32, are summarized graphically in Fig. 2. As shown in Figs. 1 and 2, the control (time 0 or un-ballooned) carotid arteries expressed undetectable and/or very low levels of mRNA for each of these IL-1 system components. Time course study revealed that the IL-1β mRNA expression was markedly induced at 6 h (29.7-fold increase over control, $p < 0.001$, n = 5) and rapidly diminished after 1 day following balloon angioplasty. No signal was detected for IL-1α mRNA expression in the carotid artery after balloon angioplasty (data not shown). The level of IL-1ra mRNA was markedly increased but peak induction (at 1 day, 400-fold increase, $p < 0.001$) was delayed compared to that of IL-1β. In addition, the elevated expression of IL-1ra was sustained up to 14 days post injury. The induction of IL-1RII mRNA was remarkably parallel with that of IL-1β, reaching a peak level at 6 h following angioplasty (100-fold increase, $p < 0.001$), whereas a significant induction of IL-1RI mRNA was not observed until 1 day post injury (14.2-fold increase, $p < 0.01$) and peaked at 3 days (20.6-fold increase, $p < 0.001$). The elevated expression of IL-1RI mRNA was extended up to 14 days after balloon angioplasty (5.6-fold increase).

Since a relative strong signal for IL-1β and IL-1ra mRNA expression was observed after balloon angioplasty, their expression profile was confirmed by Northern analysis at selected time points (Fig. 3). The Northern hybridization verified the data generated by the semi-quantitative RT/PCR.

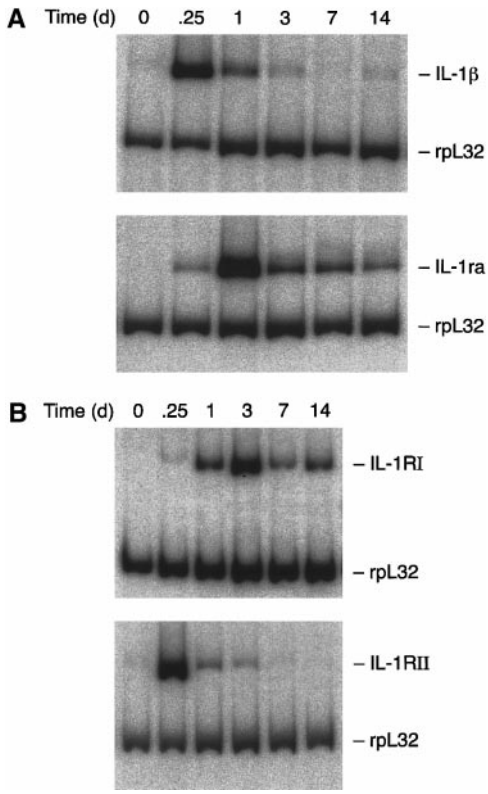

FIG. 1. RT/PCR analysis of IL-1β and IL-1ra mRNA expression (A) and IL-1RI and IL-1RII mRNA expression (B) in rat carotid artery after balloon angioplasty. RT/PCR was performed using the standard conditions as described in detail under Materials and Methods. Each sample at day 0, 0.25, 1, 3, 7, and 14 after balloon injury was pooled from three animals. The coamplified PCR products (10 μl per lane) were resolved by electrophoresis in a 6% polyacrylamide gel, dried, and autoradiographed. Representative autoradiographs show the coamplified samples of IL-1β and IL-1ra with rpL32 for 28 cycles, or IL-1RI and IL-1RII for 30 cycles.

Immunohistochemical analysis of IL-1β expression in rat carotid artery after balloon angioplasty. The temporal expression and spatial distribution of IL-1β in rat carotid artery after balloon angioplasty were examined using immunohistochemical methods. As shown in Fig. 4, no immunoreactive signal was detected in carotid artery at day 0 (Figs. 4A and 4E). Strong immunoreactivity for IL-1β was detected in the medial SMCs adjacent to the lumen (Figs. 4B and 4F) at day 1 after balloon injury. The IL-1β immunoreactive signal was also observed in some adventitial cells at day 1 after vascular injury (Fig. 4F). It is interesting to note that strong immunoreactivity for IL-1β was observed thoughout the neointimal SMCs at 14 days after angioplasty (Figs. 4C and 4G), which was sustained much longer than IL-1β mRNA. As a control, the immunoreactivity was abolished when the polyclonal anti-rat IL-1β antibodies were preincubated

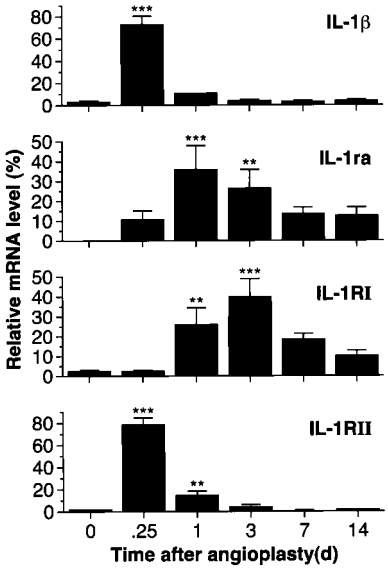

FIG. 2. Temporal expression of IL-1β, IL-1ra, IL-1RI, and IL-1RII mRNA in rat carotid artery after balloon angioplasty. The amplified DNA bands were quantitated by PhosphorImager analysis. The ratios of IL-1β, IL-1ra, IL-1RI, or IL-1RII to rpL32 in each coamplified sample were calculated and their relative levels were illustrated. Data are presented as the mean values ± standard errors of five separate experiments (15 animals) for each time point. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to controls (time = 0).

with recombinant IL-1β prior to the immunostaining (Figs. 4D and 4H).

DISCUSSION

In the present study, we examined the temporal expression of IL-1β, IL-1ra, IL-1RI and IL-1RII mRNA in rat carotid artery after balloon angioplasty. A robust induction of IL-1β mRNA and peptide was observed soon (6–24 h) after balloon angioplasty and the elevated peptide level was sustained up to 14 days. It is interesting to note that the induced expression of IL-1β is primarily located in SMCs that are actively involved in proliferation and migration, suggesting its potential role related to the neointimal formation (19). The effect

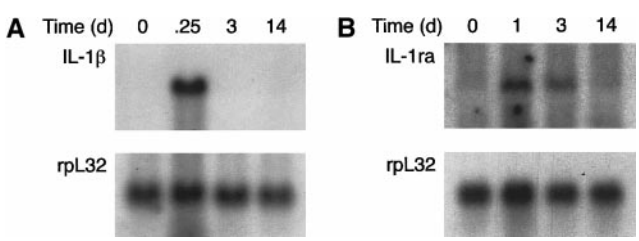

FIG. 3. Northern analysis of IL-1β (A) and IL-1ra (B) mRNA expression in rat carotid artery after balloon angioplasty. Total cellular RNA (10 μg/lane) was resolved by electrophoresis, transferred to a nylon membrane, and hybridized to the cDNA probes as indicated.

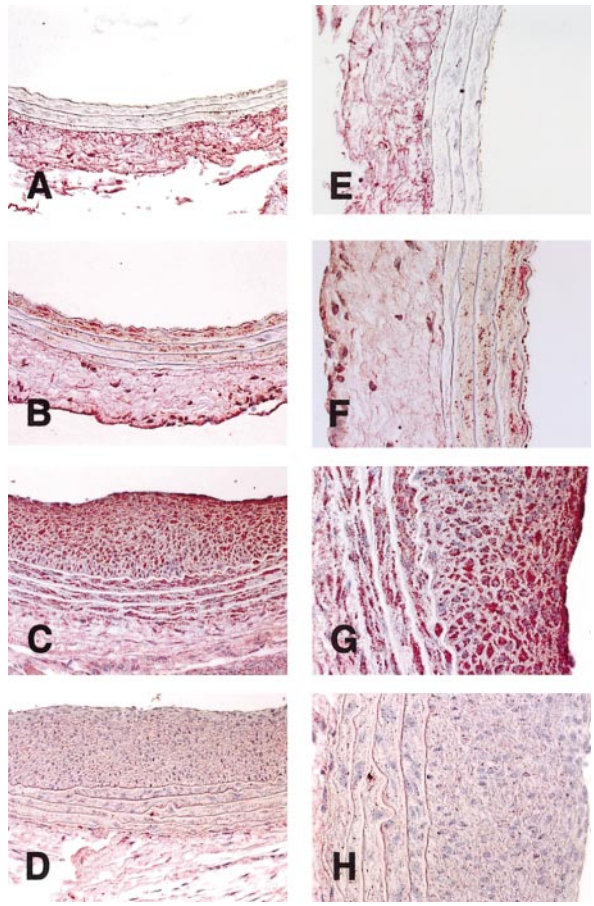

FIG. 4. Immunohistochemical detection of IL-1β expression in rat carotid artery after balloon angioplasty. IL-1β protein expression in the carotid artery was examined at day 0 (A and E), day 1 (B and F), and day 14 (C and G) following balloon angioplasty. When the anti-IL-1β antibodies were preincubated with recombinant IL-1β, the immunoreactive signal was abolished as shown in D and H for the immunostaining of rat carotid artery at day 14 after balloon injury. Magnifications: 50× for A–D and 100× for E–H.

of IL-1β on SMC proliferation and migration has been demonstrated *in vitro* (20–23), of which the mitogenic activity of IL-1β on SMCs and fibroblasts was due to the action though PDGF (21). The induced expression of IL-1β in neointima formation may involve additional functions. For example, IL-1β expression may regulate extracellular matrix expression and function (23, 24) and therefore affect cell migration. The induced expression of IL-1β may affect endothelial function in neointima formation since a concomitant induction of both the IL-1 system (present report) and the endothelin system (25) have been observed in rat carotid artery after balloon angioplasty, and the modulation of IL-1β on endothelin receptor expression and function in SMCs has been demonstrated (26). In addition, the possibility for the involvement of IL-1β in SMC apoptosis after vessel injury cannot be excluded (27, 28).

The expression of IL-1β in neointima formation has also been reported previously (29–31) but the data are

discrepant. Hancock *et al.* (29) observed a diffuse IL-1β immunostaining in the neointimal cells at limited time point (14 days) after balloon angioplasty in rat, whereas Tanaka *et al.* (30) failed to detect IL-1β signal in spite of the detection of a strong TNF-α signal in rabbit aorta after balloon injury. Another recent study (31) demonstrated that IL-1β mRNA was induced as early as 1 h and reached peak level at 6 h in porcine coronary artery after balloon angioplasty, which is in agreement with our present study in rat. In addition, the induced expression of IL-1β was observed in the neointimal cells at day 7 and in adventitial cells at day 3–14 after angioplasty in porcine (31). Overall, the temporal and spatial distribution of IL-1β expression of our present study in rat carotid artery is essentially in agreement with the previous reports in rat (29) and in porcine (31).

The expression of IL-1ra, IL-1RI and IL-1RII mRNA in neointimal formation has not been previously reported. Thus, the key novel findings of our present work are the de novo induction of the additional IL-1 system components in the carotid artery after balloon angioplasty. Each of these components is known to play a critical role in interleukin-1 functions. Of noted, IL-1ra is a naturally occurring antagonist for IL-1. The induced expression of IL-1ra mRNA immediately following IL-1β suggest that IL-1ra may interfere with IL-1β mediated effects in neointimal formation. The inhibitory role of IL-1ra on IL-1-mediated SMC proliferation could be supported by a previous report (32). It is also interesting to note that IL-1RI and IL-1RII mRNA expressions are differentially regulated following balloon angioplasty. This difference may reflect their distinct biological functions. The rapid and robust induction of IL-1RII mRNA is remarkably similar to that of IL-1β. Because IL-1RII can readily bind IL-1β but does not stimulate IL-1-mediated signal transduction (3), the induced expression of IL-1RII may provide a natural compensatory mechanism to block IL-1β action prior to the induction of IL-1ra after balloon injury. In contrast, a slightly delayed expression of the functional receptor IL-1RI immediately following IL-1β induction correlates well with IL-1β action in neointimal formation.

In addition to IL-1β, IL-1ra and IL-1Rs, other members of IL-1 system have been identified and demonstrated to play important roles in IL-1 mediated actions which includes IL-1α, interleukin-1 converting enzyme (ICE or caspase-1) and IL-1 receptor accessory protein (IL-1R-AcP) (3). The expression of IL-1α mRNA was evaluated but no signal was detected in neointimal formation (data not shown). ICE is an intracellular protease and is a member of cysteine protease family. The key function of ICE is to cleave pro-IL-1β to form the active form of IL-1β. The parallel expression of ICE and IL-1β was observed in the porcine coronary artery after balloon angioplasty (31), suggesting that IL-1β induced after balloon injury is biological active. IL-1R-AcP is an Ig-like domain containing peptide and plays a role in IL-1 signal transduction by facilitating the binding of IL-1 to IL-1RI (3). The expression of IL-1R-AcP in neointimal formation remains to be evaluated.

In conclusion, the present study revealed the kinetic induction profiles for IL-1β, IL-1ra, IL-1RI, and IL-1RII mRNA in rat carotid artery after balloon angioplasty, though their protein profiles remain to be elucidated. These data suggest the potential active involvement of these IL-1 components (especially the concomitant induction of IL-1β and IL-1RI) in neointimal formation. The upregulation of the endogenous IL-1 antagonists (IL-1ra and IL-1RII) after balloon angioplasty may attenuate the biological effect of IL-1β. The final balance between the agonists and antagonists may determine the pathogenesis (i.e., neointimal formation) in response to vascular injury, although other factors such as PDGF, bFGF, TGFβ and TNF-α are also induced after vascular injury cannot be excluded. In addition, the exact role(s) of IL-1 system in neointimal formation remains to be investigated prior to the establishment of it as a therapeutic target for restenosis.

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