Effects of histamine and interleukin-4 synthesized in arterial intima on phagocytosis by monocytes/macrophages in relation to atherosclerosis

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Abstract **We investigated the localization of histidine decarboxylase (HDC), which is the rate-limiting enzyme that generates histamine from histidine, in human aorta/coronary artery. RT-PCR and immunohistochemical staining revealed that the HDC gene was expressed in monocytes/macrophages and T cells in the arterial intima but not in smooth muscle cells in either the arterial intima or the media. A luciferase promoter assay with U937 and Jurkat cells demonstrated that interleukin-4 (IL-4) inhibited the expression of the HDC gene. In contrast, among a scavenger receptor family, IL-4 as well as histamine up-regulated U937 cells to express the LOX-1 gene but not the SR-A gene, which genes encode receptors that scavenge oxidized lipids. These findings suggest that histamine synthesized in the arterial wall participates in the initiation and progression of atherosclerosis and that IL-4 can act as an important inhibitory and/or stimulatory factor in the function of monocytes/macrophages modulated by histamine in relation to the process of atherosclerosis.**

Key words: Histamine; Interleukin-4; Histidine decarboxylase; LOX-1; Atherosclerosis; U937 cell; Jurkat cell; Promoter assay

1. Introduction

Histamine induces reversible constriction of vascular and non-vascular smooth muscle cells (SMC) and causes microvascular vasodilatation and increased permeability of venules [1,2], endothelial cells contraction [3], gap formation [4], allergic reactions [5], and modulation of gastrointestinal function [6] and of neurotransmission [7]. Histamine is released from dense granules in mast cells and basophils, which cells are located within the connective tissue and are prevalent along the mucosal surface of the lungs and gastrointestinal tract and in the dermis of the skin [8]. Three types of histamine-specific receptors are known, H1, H2, and H3 [9–11], and histamine acts on the vasculature by binding mainly to specific H1 receptors in the vascular wall.

In previous studies [12,13], we demonstrated that histamine synthesized by mast cells is involved in the processes of remodeling of the arterial intima via its ability to stimulate the proliferation of SMC and production of matrix metalloproteinases (MMPs) and that the density of histamine receptors in SMC in the aorta and coronary artery increased in relation to the progress of atherosclerosis. In addition, we also reported that monocytes/macrophages derived from peripheral blood expressed histamine receptors and that during maturation from monocytes to macrophages, a switch of histamine receptor expression from H2 to H1 took place [14]. These findings led us to consider that the large number of histamine receptors expressed widely participate not only in pathological but also in physiological events and that three specific receptors share functions of histamine, even within the vascular wall. Indeed, an increased number of mast cells have been found in the arterial intima containing advanced atherosclerotic foci [15] and in the adventitia of the involved artery in patients with coronary spasms and associated vasospasm [16].

Histamine is produced by histidine decarboxylase (HDC), which is the rate-limiting enzyme that generates histamine from histidine through a single enzymatic reaction [17]. In addition to being produced by mast cells/basophils, histamine is also synthesized in T lymphocytes and monocytes/macrophages [18,19], which are important cell types responsible for the progression of atherosclerosis [20–23]. Since compared with the relatively lower number of mast cells, numerous macrophages and T lymphocytes are commonly aggregated in and/or around an atheroma, these cell types may be more important sources of histamine than mast cells in atherosclerotic foci. Therefore, we investigated the expression, localization and regulation of the HDC gene in the human arterial wall to check other possible sources for histamine in the site.

On the other hand, it is well known that cytokines, which are produced by infiltrating monocytes/macrophages and Th1 and Th2 type lymphocytes, play an important role in the pathogenesis of allergic inflammation in the arterial wall [21,22,24]. Among them, we found that interleukin-4 (IL-4) inhibits the growth of SMC and stimulates the production of MMPs [23], suggesting that IL-4 synthesized by T cells in the arterial intima may participate in the regression and prevention of atherosclerosis. In the present communication, in addition to examining the localization of HDC in the arterial intima, we also discussed the effects of histamine and IL-4 on

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the function of monocytes/macrophages in relation to atherosclerosis.

2. Methods and materials

2.1. Chemicals

Tetradecanoylphorbol 13-acetate (TPA) and phytohemagglutinin (PHA) were obtained from Sigma (St. Louis, MO, USA). Human recombinant IL-4 was from Peprotech (London, UK). Lipofectin reagent for plasmid transfection (DIMRIE-C) was purchased from Gibco BRL (Grand Island, NY, USA).

2.2. Cell culture

The human T cell leukemic cell line (Jurkat cells) and a human monocytic leukemic cell line (U937 cells) used were obtained from ATCC and maintained in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) with 10% fetal calf serum (FCS; ICN, Irvine, CA, USA), 100 U/ml of penicillin, and 2.5 μg/ml of fungizone at 37°C in an atmosphere of 95% air and 5% CO_2 in the presence or absence of histamine, IL-4, TPA and/or PHA.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs extracted from Jurkat or U937 cells with Trizol reagent (Gibco BRL) were subjected to the reverse transcriptase reaction and used as templates for PCR amplification of HDC cDNA. Autopsied materials from human aortas were also used for the RT-PCR, as described previously [14], which used specific primers (forward 5′-CAAGCACATGTCAGACATGG-3′ and reverse 5′-TGAACAGGAAGGAGGACAGA-3′) of the human HDC gene (GenBank M60445) for the amplification. Total RNA (5 μg) was exposed to SuperScript II RNase H reverse transcriptase (1000 U; Gibco BRL) in the presence of random primer (500 ng), dNTP (0.9 mM each), and first-strand buffer in a total volume of 50 μl. The reaction was allowed to continue for 1 h at 37°C. A 1-μl aliquot of each reaction mixture was then subjected to PCR using specific primers. Denaturation, annealing and extension temperatures and time were 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min, respectively, for 35 cycles. On completion of the PCR, the amplified DNA fragments of RT-PCR (589 bp from +1225 to +1813) were subcloned in a pGEM-T Easy vector (Promega, Madison, WI, USA), and the purified DNA was analyzed with an ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) to confirm their specific sequences.

2.4. Northern blotting

Total RNAs (10 μg) were extracted from cultured cells stimulated with TPA and/or PHA for 24 h in the presence of IL-4 and electrophoresed in 1% agarose/formaldehyde denaturing gel, blotted onto Geenscreen plus (DuPont, Boston, MA, USA), and prehybridized for 30 min at 68°C with QuickHyb Hybridization solution (Stratagene, La Jolla, CA, USA). The amount of the HDC transcripts was evaluated by hybridization with ^{32}P-labeled specific probes (589-bp RT-PCR product) in the hybridization solution. After extensive washing to remove background non-specific binding, the membrane was exposed to an X-ray film overnight at −80°C.

2.5. Cloning of HDC, LOX-1, and SR-A promoter regions

Based on the reported sequence data of the HDC (GenBank D16583), LOX-1 (GenBank AB021922), and SR-A (GenBank D13263) promoter regions, the cloning of 361-, 1063-, and 677-bp fragments corresponding to nucleotides −240 to +121, −1017 to +36, or −630 to +47, respectively, was conducted following PCR using genomic DNA of the Jurkat cells or U937 cells with specific primers (HDC: forward 5′-*Sac*I+TATTGTCTGAATCCAGGGGCA-3′, reverse 5′-*Hin*dIII+CCTTCTCCACAGATGGACACGC-3′; LOX-1: forward 5′-*Nhe*I+ACTTATGGGTCTCTCATGTAAGCG-3′, reverse 5′-*Xho*I+TGAAGCAGTCACGAACTTCAA-3′; SR-A: forward 5′-*Mlu*I+AGAGCTCCTGACCTCAAGTGATCC-3′, reverse 5′-*Hin*dIII+CCTCATAGTATTTCAGCATCTG-3′). After each sequence had been confirmed, the fragment was ligated to a luciferase reporter vector, pGL3-basic, at each restriction enzyme site (pHDC-LucWt, pLOX-1-Luc, and pSR-A-Luc, respectively). Two mutated pHDC-LucWt constructs were generated by self-ligation after *Apa*I (located at +16) digestion and Klenow treatment (pHDC-LucM2), and conversion of CT to GA (located at from +2 to +6) by site-directed mutagenesis (pHDC-LucM1). The structures of the luciferase reporter constructs are presented in Fig. 4A.

2.6. Transfection and luciferase assay

The Jurkat cells were transfected by a method of lipid-mediated gene transfer according to the manufacturer's manual (DIMRIE-C reagent, Gibco BRL). Briefly, the Jurkat cells (1×10^6) were incubated with 3 μg of reporter plasmid along with 0.2 μg of SV40-β-galactosidase (β-gal) plasmid and 5 μl of the DIMRIE-C reagent in 0.4 ml of OMTI-MEM medium (Gibco BRL). After a 5-h incubation, 0.6 ml of RPMI 1640 with 5% FCS was added (final serum concentration was 3%), and the cells were further incubated for 24 h with or without TPA (10 ng/ml) and PHA (1 μg/ml). IL-4 was added at the desired time of the incubation. For U937 cells (1.4×10^7), 20 μg of reporter constructs with 0.5 μg of SV40-β-gal were used for transfection by electroporation (950 μF, 300 kV). After 8 h incubation with TPA and/or IL-4, the cells were lysed with a lysis buffer (Tokyo Ink, Tokyo, Japan) and mixed with luciferase substrate (Toyo Ink) for measurement of the luciferase activity. To decrease the variation in the transfection efficiency, we transfected the cells in single batches for each reporter plasmid, which were then separated into three wells for triplicate study. Further, β-gal activity was measured to monitor the transfection efficiency. Transcriptional activities were expressed as fold activation relative to that of sham-treated cells transfected with promoterless pGL3-basic vector.

3. Results and discussion

3.1. Expression of the HDC gene in the human aortic wall

To investigate HDC gene expression in human aortas, we extracted total RNA samples from the aortic wall of autopsy cases and applied them to RT-PCR, as reported previously [23]. As shown in Fig. 1, the message of the HDC gene transcription was detected in the normal intima (Fig. 1, lane N), fatty streak or fibrous plaque (Fig. 1, lane F), and atheromatous foci (Fig. 1, lane A), whereas in lesions that had markedly progressed and were accompanied by calcification and ulceration, the expression was sometimes not detectable. The RNAs extracted from human aortic media did not contain HDC mRNA, either. These data reveal that the HDC gene

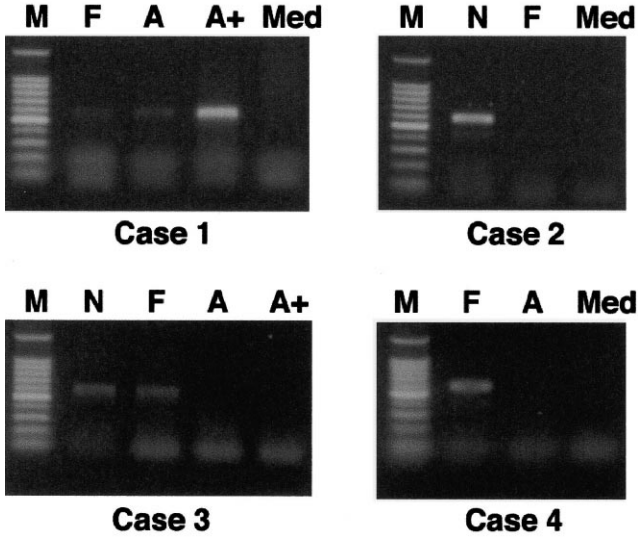

Fig. 1. RT-PCR detection of HDC gene expression in the human aorta. The total RNA was extracted from human aortas (four autopsy cases) and used for RT-PCR examination. In all cases, RT-PCR product of the HDC gene was detected in normal or slightly thickened intima (lane N), fatty plaque (lane F), and/or atheromatous area (lane A) or that with calcification (lane A+) but not in the media (lane Med). Lane M: size markers.

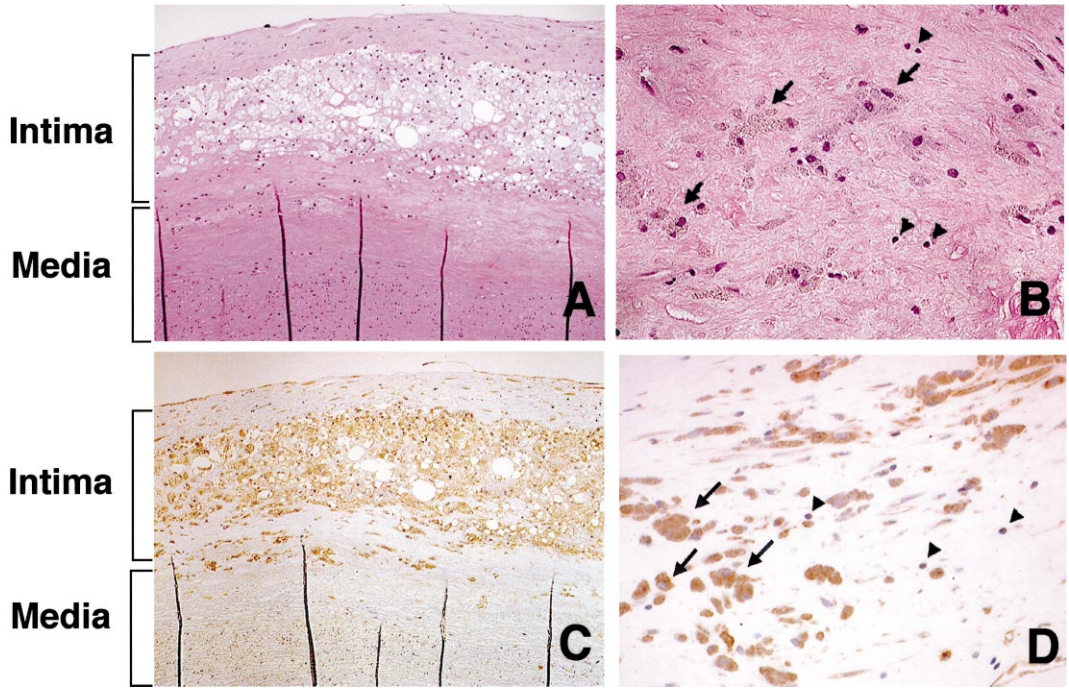

Fig. 2. Immunohistochemical staining of HDC in atherosclerotic foci in both human coronary artery. Light microscopic photographs show atheromatous plaques (A and B) containing large numbers of macrophages (arrows) and a lesser number of lymphocytes (arrowheads) in human aortic intima (hematoxylin–eosin-stained slides). Immunohistochemical staining revealed strongly positive staining for HDC in the cells forming the atheromatous foci but not in the medial smooth muscle cells (C). At higher magnification, HDC-positive cells, i.e. macrophages (arrows) and lymphocytes (arrowheads), are more clearly seen (D). Magnification: 30× for A and C and 150×for B and D.

is expressed in the aortic intima in relation to the progression of atherosclerosis.

3.2. Localization of HDC in the human arterial wall

Immunohistochemical staining with antibodies against HDC, CD68 (a macrophage marker), αSMA (a smooth muscle cell marker), and CD45RO (a T cell marker), revealed that the production of HDC occurred mainly in monocytes/macrophages and in a lesser number of T cells in the intima of the aorta (Fig. 2) and coronary artery. HDC could not be detected in SMC in either the intima or the media. Identification of these HDC-positive cell types was confirmed by the result that they also reacted positively with antibodies against CD68 or CD45RO but not with anti-αSMA (not shown). These findings confirm those of the RT-PCR experiments and suggest that the transcription and translation of the HDC gene in the arterial intima participate in atherosclerotic processes in the arterial wall. Unlike mast cells, monocytes and T cells do not have many granules containing various kinds of inflammatory mediators. Histamine synthesized in these cell types could be directly and continuously secreted and could function in an autocrine and/or paracrine way in the site.

3.3. Expression of the HDC gene in monocytes/macrophages and in T cell lines

Next, to address which types of cells potentially express the HDC gene and to confirm the results of the immunohistochemical staining, we used T cells (Jurkat) and U937 cells for RT-PCR and Northern blot experiments. RT-PCR demonstrated that Jurkat cells did not express the HDC gene under normal culture conditions (Fig. 3A, lane 1). Upon stimulation of Jurkat cells with both TPA (10 ng/ml) and PHA

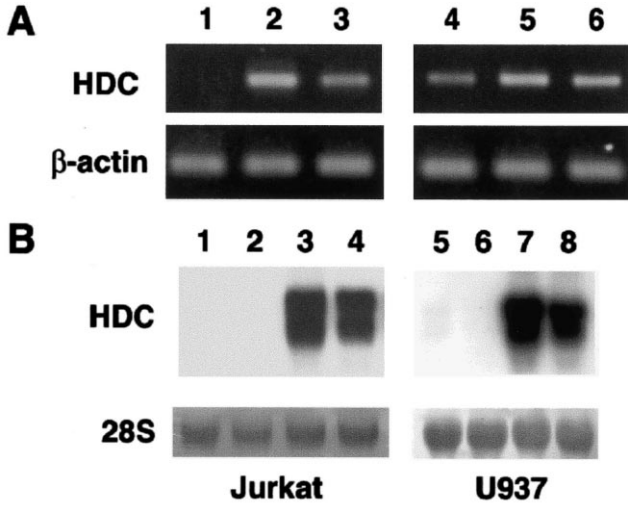

Fig. 3. Expression of the HDC gene in cultured cells. A: RT-PCR analysis reveals HDC gene expression in Jurkat and U937 cells. Jurkat cells did not express the HDC gene under normal culture conditions (lane 1), whereas in U937 cells, a certain amount of its expression was detected under normal culture conditions (lane 4). Upon stimulation with 10 ng/ml TPA and 1 μg/ml PHA, Jurkat cells appeared to express the HDC gene dramatically (lane 2), and the addition of IL-4 suppressed this up-regulation (lane 3). In U937 cells, IL-4 (lane 6) also reduced up-regulation of HDC gene expression caused by TPA (lane 5). B: Northern blot analysis of HDC mRNA in Jurkat and U937 cells. No certain amount of HDC mRNA was detected in either Jurkat (lane 1) or U937 cells (lane 5) under normal culture conditions, or even after the addition of 5 ng/ml of IL-4 (lanes 2 and 6). TPA (10 ng/ml) and PHA (1 μg/ml) or TPA alone stimulated transcription of the HDC gene in both Jurkat and U937 cells (lanes 3 and 7, respectively). The addition of IL-4 (5 ng/ml) reduced the up-regulation of the HDC gene transcription by TPA/PHA (lanes 4 and 8).

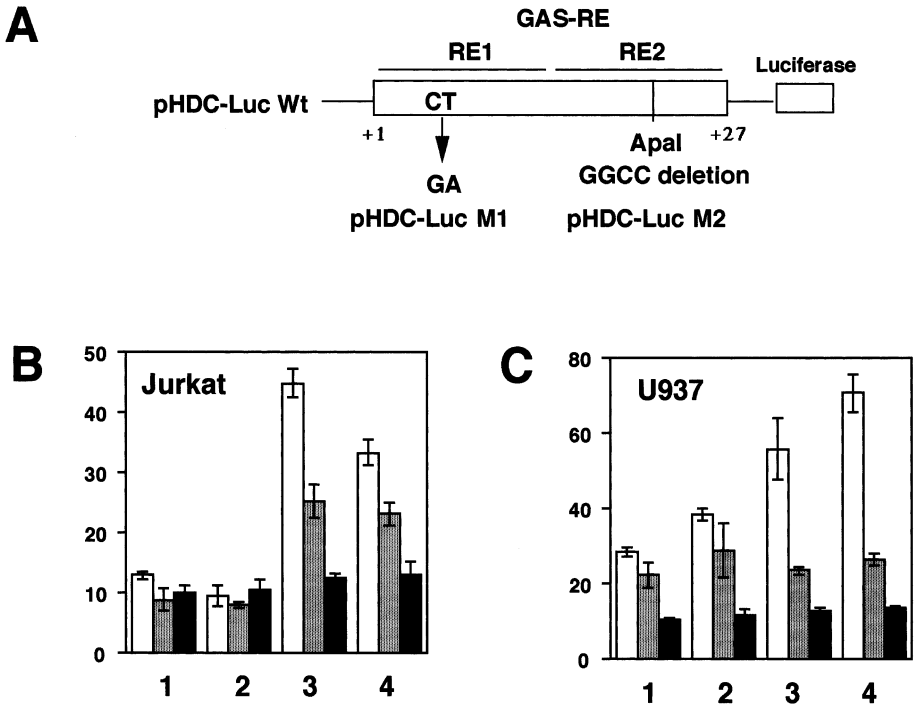

Fig. 4. Luciferase reporter assay of the HDC gene in Jurkat or U937 cells. A: Schematic presentation of HDC promoter constructs, which contain GAS-RE1 and GAS-RE2. pHDC-LucWt: 361 bp, from the *Sac*I site (−240) to the *Hin*dIII site (+121); pHDC-Luc M1: mutant with a two-nucleotide substitution of CT to GA at the core motif in RE1; pHDC-LucM2: deletion of four nucleotides (GGCC) from the core motif (*Apa*I restriction enzyme site) in RE2. B: Luciferase reporter assay of the HDC gene in Jurkat cells. Open column: pHDC-LucWt; hatched column: pHDC-LucM2; closed column: pHDC-LucM1; 1: untreated, basal levels; 2: treated with IL-4 (10 ng/ml); 3: treated with TPA (10 ng/ml) and PHA (1 μg/ml); 4: treated with IL-4 (10 ng/ml)+TPA (10 ng/ml)/PHA (1 μg/ml). C: Luciferase reporter assay of the HDC gene in U937 cells. Open column: pHDC-LucWt; hatched column: pHDC-LucM2; closed column: pHDC-LucM1; 1: untreated, basal levels; 2: treated with IL-4 (10 ng/ml); 3: treated with TPA (10 ng/ml); 4: treated with IL-4 (10 ng/ml)+TPA(10 ng/ml).

(1 μg/ml) for 16 h, HDC gene transcription was enhanced dramatically (Fig. 3A, lane 2), whereas this up-regulation was suppressed by addition of IL-4 (5 ng/ml) with the TPA and PHA (Fig. 3A, lane 3). Except for a certain amount of the HDC mRNA transcripts in untreated U937 cells, the data on U937 cells were similar to those on Jurkat cells.

To confirm these results further, we performed Northern blotting. With stimulation by both TPA and PHA for 16 h, the HDC mRNA was extensively expressed in the Jurkat cells (Fig. 3B, lane 3), whereas no mRNA was detected in non-treated cells (Fig. 3B, lane 1) or in cells treated with IL-4 (Fig. 3B, lane 2) for 16 h. In contrast, treatment with IL-4 for 16 h apparently suppressed the up-regulation of the HDC mRNA by TPA and PHA (Fig. 3B, lane 4). On the other hand, in Northern blotting of U937 cells, HDC gene expression was pronounced by TPA alone (Fig. 3B, lane 7), and the addition of IL-4 suppressed the TPA-stimulated HDC gene expression (Fig. 3B, lane 8).

These data confirm that the HDC gene is expressed by activated T cells and by a monocyte/macrophage lineage in the process of differentiation and that IL-4 is an inhibitory factor, even with respect to the expression of the HDC gene.

3.4. Luciferase reporter assay

To address whether IL-4 transcriptionally down-regulates the HDC gene in the Jurkat cells, we constructed pHDC-LucWt. As shown in Fig. 4A, the pHDC-LucWt construct included the minimal enhancer element(s) originally responsive to gastrin and TPA (gastrin-responsive elements, GAS-RE) [25]. In our preliminary study, the pHDC-LucWt construct in Jurkat cells was sufficiently stimulated by TPA/PHA. Maximal transcriptional activity from the pHDC-LucWt reporter construct by stimulation with TPA/PHA was obtained at 24 h after transfection of the Jurkat cells (data not shown), and so further studies were performed with a fixed time of stimulation with TPA/PHA (24 h) and various times of IL-4 treatment. After transfection with the reporter constructs by lipid-mediated gene transfer, the Jurkat cells were stimulated with 10 ng/ml of TPA and 1 μg/ml of PHA for 24 h with a final 12-h treatment with 10 ng/ml of IL-4, and then the luciferase activity was measured.

IL-4 stimulation alone did not have any effect on the promoter activity of any of the constructs (Fig. 4B, lane 2). Whereas TPA/PHA stimulation enhanced the promoter activities from pHDC-LucWt (four-fold over the basal activity) and pHDC-LucM2 (two-fold), pHDC-LucM1 had no enhanced transcriptional activity over the basal level (Fig. 4B, lane 3). By treatment with IL-4 at 12 h before cell harvesting for the luciferase assay, HDC promoter activity was reduced to 75% with pHDC-LucWt, but no reduction was detected in pHDC-LucM2 (Fig. 4B, lane 4). In the U937 cells, the maximal promoter activity was observed at 8 h after electroporation when stimulated with TPA, whereas treatment with IL-4 did not reduce the promoter activity from the pHDC-LucWt construct (Fig. 4C). The activity of the pHDC-LucM2 construct had the same basal level as pHDC-LucWt, but no IL-4 or TPA response was observed. In the cells transfected with pHDC-LucM1, the basal promoter activity was less than 50%

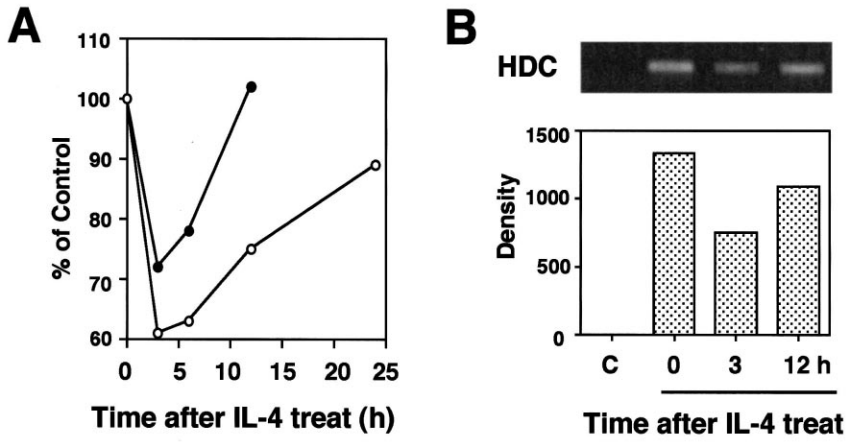

Fig. 5. Time course of effect of IL-4 on luciferase activity and on RT-PCR product in Jurkat cells. A: After transfection with pHDC-LucWt or M2, the cells stimulated by TPA and PHA were incubated with 10 ng/ml of IL-4, and then luciferase activity was measured. Note that 3–6 h after incubation with IL-4, luciferase activity markedly decreased and then recovered gradually (open circle: pHDC-LucWt; closed circle: pHDC-LucM2). B: Upon the stimulation of Jurkat cells with 10 ng/ml of IL-4, RT-PCR revealed results that paralleled those of the luciferase reporter assay.

of that for pHDC-LucWt, and the low activities remained following IL-4 and/or TPA treatments.

In the time course of the effect of IL-4 in Jurkat cells, the TPA/PHA-induced promoter activities from pHDC-LucWt and pHDC-LucM2 constructs showed 60–70% reduction 3 h after treatment with IL-4 and recovered to 100% and 90% of the control level 12 and 24 h later, respectively (Fig. 5A). The result of RT-PCR paralleled those of the luciferase reporter assay (Fig. 5B).

These analyses show that IL-4 suppressed transcriptionally HDC gene expression in Jurkat cells. This down-regulation of HDC mRNA by IL-4 was dose-dependent in both RT-PCR and Northern blot analyses (data not shown).

The reporter gene for HDC containing GAS-RE was composed of RE1 and RE2 regions. Our data revealed that RE1 is more responsible for the expression of the gene than RE2, as reported previously [26]. The deletion analysis of the human HDC gene promoter region showed that the *cis*-acting DNA sequence responsible for gastrin-mediated transactivation is located in a region (+2 to +24) downstream of the transcriptional start site (+1), which is called the gastrin-response element (GAS-RE), by which the effect of phorbol ester might be mediated.

3.5. Reporter assay of scavenger receptors

Next, we investigated whether in situ histamine production is involved in the function of monocytes/macrophages that migrated into the arterial wall. The scavenger activity toward modified lipid that accumulates in the arterial intima is one of the most important functions of monocyte/macrophages. Therefore, we constructed the promoter regions of the type A scavenger receptor (SR-A) and the LOX-1 genes to check the effects of histamine and IL-4 on the promoter activity of these genes. As shown in Fig. 6, in untreated U937 cells or in

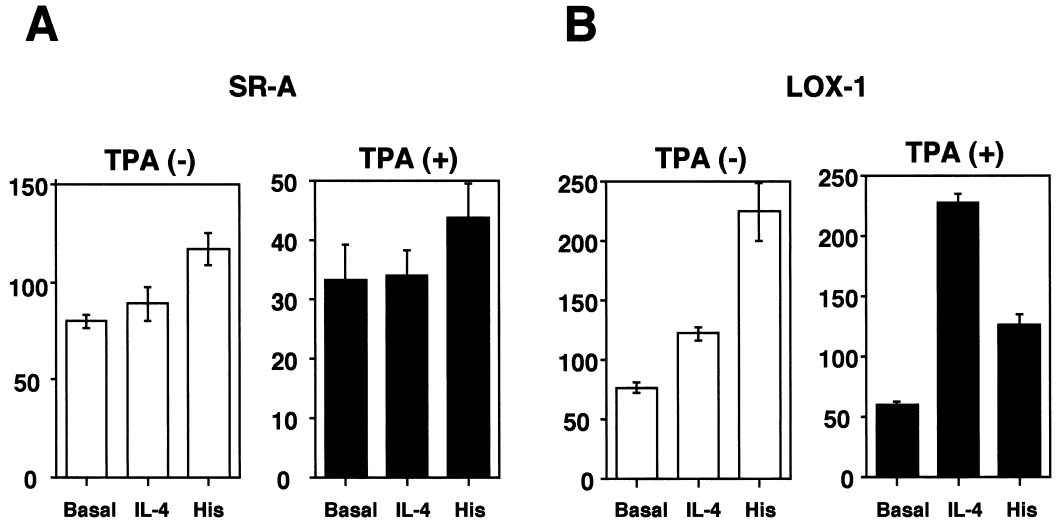

Fig. 6. Effects of histamine and IL-4 on function of macrophages. Luciferase reporter assay of the SR-A and the LOX-1 gene in U937 cells was conducted. After transfection with pSR-A-Luc or pLOX-1-Luc by electroporation (Gene Pulsar II, Bio-Rad, Hercules, CA, USA), the cells were stimulated with 100 μM histamine or 10 ng/ml of IL-4 for 8 h, and then the luciferase activity was measured. The promoter activity for pSR-A-Luc failed to respond to either histamine or IL-4 in U937 cells, even after TPA treatment. Histamine and IL-4, however, stimulated the cells to up-regulate the promoter activity of the LOX-1 gene, even after incubation with 10 ng/ml TPA.

those treated with TPA for 24 h followed by a 48-h starvation, histamine up-regulated markedly and dramatically the promoter activity of the LOX-1 gene, which encodes a lectin-like oxidized low-density lipoprotein (LDL) receptor, whereas it failed to stimulate the activity of the SR-A promoter. Unexpectedly, IL-4 also enhanced the promoter activity of the LOX-1 gene in particular, in TPA-treated cells, suggesting that histamine enhanced the LOX-1 gene in monocytes, whereas IL-4 stimulated mature U937, i.e. macrophages, to express the gene. From these data taken together, we think that histamine and IL-4 biosynthesized in the arterial intima stimulate monocyte/macrophages to phagocytose oxidized LDL, resulting in the formation of foam cells.

4. Discussion

Our data demonstrated that via HDC activity, monocytes/macrophages and T cells have the ability to biosynthesize and to secrete histamine in an autocrine and/or paracrine manner in the arterial intima. Based on our data, histamine synthesized in the site can stimulate monocytes/macrophages to express the LOX-1 gene, indicating that histamine acts on monocytes/macrophages to phagocytose and accumulate oxidized LDL, which accumulation is an essential event of atherosclerosis. We reported previously that histamine stimulates SMC to proliferate and to produce MMPs via the H1 receptor [13] and that during maturation of the cells of the monocyte/macrophage lineage, dominant expression of the histamine receptor changes from H2 to H1 [14]. Since the effect of histamine on up-regulation of the LOX-1 gene was stronger in untreated U937 cells than in cells treated with TPA, the histamine H2 receptor should participate in the up-regulation of the LOX-1 gene in these cells. Our RT-PCR experiments revealed that the SR-A gene, but not the LOX-1 gene, was constitutively expressed under normal culture conditions (data not shown). Regulation of the LOX-1 gene seems to be more critical and complex as compared with that of other scavenger receptors such as SR-A and B [27]. Therefore we think that among several scavenger receptors, LOX-1 may especially be involved in the function of monocytes/macrophages in pathological processes including atherosclerosis.

We reported earlier an inhibitory effect of IL-4 on proliferation of SMC and its stimulatory effect on MMP production [23]. Similarly, the present study showed that IL-4 acts also as a suppressor of histamine synthesis, and as a stimulator of LOX-1 gene expression. Unlike untreated U937, upon activation with TPA, the effect of IL-4 on LOX-1 gene expression was more pronounced. Consequently, we consider that mature monocytes, i.e. macrophages, could be more sensitive to IL-4 to express the LOX-1 gene. Interestingly, it was reported that histamine can inhibite T cells to produce IL-4 [24,28]. Thus, there seems to be a complicated interaction between monocyte/macrophages and T cells via histamine and IL-4 production in the arterial intima.

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