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Angiotensinogen production by rat hepatoma cells is stimulated by B cell stimulatory factor 2/interleukin-6

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Angiotensinogen has been identified as one of the acute-phase reactants. In vitro studies were carried out using the Reuber H35 hepatoma cell line to identify the species of cytokines contributing to the increased synthesis of angiotensinogen in the liver. Angiotensinogen secretion by H35 cells was maximally increased 4-fold by the addition of 10^{-7} M dexamethasone. Under this condition, angiotensinogen secretion was further stimulated by B cell stimulatory factor 2/interleukin-6 (IL-6, 50 U/ml), but not by interleukin-1 or interferon-α. In the absence of glucocorticoid, IL-6 did not affect angiotensinogen secretion by H35 cells, indicating that the presence of glucocorticoid is required for the stimulatory activity of IL-6. These results suggest that IL-6 is a mediator responsible for the increased synthesis of angiotensinogen in the liver during acute inflammation.

Angiotensinogen; Cytokine; Interleukin-6; Acute-phase reactant; Inflammation

1. INTRODUCTION

Angiotensinogen is a plasma glycoprotein, predominantly synthesized in the liver, that serves as the precursor of the decapeptide angiotensin I formed by the limited proteolytic action of renin. Angiotensin I is further hydrolyzed by a converting enzyme to angiotensin II, the biologically active molecule that plays an important role in the regulation of blood pressure [1].

Physiological alterations such as volume depletion and a low-sodium diet, as well as insulin, thyroxine, estrogen and glucocorticoid treatment, increase the circulating levels of angiotensinogen in the rat [1-3], and increased levels of angiotensinogen mRNA induced by these hormones have been confirmed by in vitro experiments using rat hepatoma or pancreatic islet cell lines [4,5]. In addition to these physiological factors, Kageyama et al. [6] have demonstrated that the induction of acute inflammation in the rat causes a significant elevation of the liver angiotensinogen mRNA level. Subsequently, we demonstrated that the plasma concentration of angiotensinogen was increased in rats by the induction of acute inflammation [7]. Elevated levels of plasma angiotensinogen have also been observed in humans with acute inflammatory diseases [8]. These changes in plasma concentration of angiotensinogen following the induction of acute inflammation identify this protein as a member of the class of proteins designated 'acute-phase reactants' [9]. Since the acute-phase response of angiotensinogen was found in neither adrenalectomized [7] nor hypophysectomized rats [10], it was suggested that the rise in plasma angiotensinogen under inflammatory conditions is secondary to increased adrenocortical secretion. However, since no changes in plasma glucocorticoid level were observed in rats prior to elevation of plasma angiotensinogen by lipopolysaccharide-induced inflammation [10], we have suggested that

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Abbreviations: IL-1, interleukin-1; IL-6, B cell stimulatory factor 2/interleukin-6; IFN-α, interferon-α; HSF, hepatocyte-stimulating factor

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glucocorticoid is not a direct mediator for the acute-phase response of angiotensinogen. In addition to these findings, we observed that implantation of leukocytes collected from rats with acute inflammation caused an elevation of the plasma angiotensinogen level in normal rats [10]. This evidence suggests that the acute-phase response of angiotensinogen results from increased synthesis of angiotensinogen in the liver stimulated by certain leukocyte-derived factor(s), and that glucocorticoid is essential for its stimulatory activity on hepatocytes.

Factors released from leukocytes control many of the acute-phase responses. It was found that activated monocytes/macrophages from humans and rodents synthesized and released components which, when added to cultured hepatocytes, were able to induce most of the acute-phase responses in plasma protein production [11–13]. These activities have been attributed to the action of cytokines including hepatocyte-stimulating factor (HSF), interleukin-1 (IL-1), tumor necrosis factor and interferon-α (IFN-α) [14–17]. Of these cytokines, HSF is known to be a major mediator of the acute-phase response [18] and has recently been identified as B cell stimulatory factor 2/interleukin-6 (IL-6) [19,20], originally known as a T cell-derived interleukin inducing the final maturation of B cells into antibody-forming cells [21,22], IFN-β_2, 26 kDa protein or hybridoma-plasmacytoma growth factor [23].

In order to identify and study the mode of action of leukocyte-derived factors involved in the acute-phase response of angiotensinogen, we used the Reuber H35 hepatoma cell line originating from the rat, because these cells have been proved to be an excellent system for the study of angiotensinogen production and its regulation by glucocorticoid [4,24]. Here, we demonstrate that IL-6 has stimulatory activity on angiotensinogen production by H35 cells, and that its effect requires the presence of glucocorticoid.

2. MATERIALS AND METHODS

2.1. *Cytokines*

Human IFN-α (2×10^8 IU/mg) and recombinant human IL-1α (2×10^7 U/mg) were donated by Hayashibara Biochemical Laboratories (Okayama) and Dainippon Pharmaceutical (Osaka), respectively. Human recombinant IL-6 (5.2×10 U/mg) was kindly supplied by Drs T. Hirano and T. Kishimoto (Institute for Molecular and Cellular Biology, Osaka University, Osaka). One unit of the IL-6 preparation was as defined by Hirano et al. [21].

2.2. *Purification of angiotensinogen*

Rat plasma angiotensinogen was purified to homogeneity according to Bouhnik et al. [24]. From 260 ml of plasma from bilaterally nephrectomized rats, 5.9 mg angiotensinogen was finally obtained. The purified angiotensinogen yielded 24.2 μg angiotensin I/mg after treatment with hog renin. This is quite close to the theoretical value of 22.7 expected for an angiotensinogen with an M_r of 57 000. SDS-polyacrylamide gel electrophoresis of the purified preparation gave two protein bands corresponding to M_r values of 56 000 and 60 000, identical to those of a homogeneous preparation of rat angiotensinogen reported by Hilgenfeldt and Hackenthal [25].

2.3. *Radioimmunoassay of angiotensinogen*

Angiotensinogen was measured by radioimmunoassay (RIA) using ^{125}I-labeled angiotensinogen and rabbit antibody against rat angiotensinogen. ^{125}I-labeled angiotensinogen was prepared by the chloramine-T method [26] and separated from free ^{125}I by Sephadex G-50 chromatography. At a dilution of 1:60 000, the antiserum bound approx. 40% of the tracer antigen. The RIA was carried out in 0.1 M Tris-HCl (pH 7.4), containing 0.2% gelatin and 0.01% neomycin sulfate. The incubation mixture contained 50 μl ^{125}I-angiotensinogen, 50 μl diluted (1:20 000) antiserum and 50 μl standard (2–500 ng) or sample. Following incubation at 4°C for 18 h, tracer that had bound to antibody was separated using Zysorbin (Zymed, San Francisco, CA) as described [27], and then counted in a gamma-counter. A standard curve was obtained by plotting the percentage of initial binding against the angiotensinogen standard. A series of dilutions of culture medium obtained by 48 h culture of H35 cells with 10^{-7} M dexamethasone gave a line parallel with the standard curve. Angiotensinogen present in fetal calf serum did not cross-react with the antibody. This RIA permitted the detection of as little as 10 ng/ml angiotensinogen.

2.4. *Culture experiments*

Reuber H35 cells [28] were kindly supplied by Dr H. Kawano (Kobe-Gakuin University, Kobe), and cultured in 6-well plates with Dulbecco's modified Eagle's medium (Nissui, Tokyo) containing 10% fetal calf serum (FCS). The medium was replaced by fresh medium every 2 days until cultures approached confluence. 2 days after reaching confluence, the medium was replaced by one supplemented with 5% FCS depleted of steroid hormones (steroid-free medium) [29]. Following 24 h culture, the medium was removed and replaced with 2 ml fresh steroid-free medium containing cytokines and/or dexamethasone (Nacalai Tesque, Kyoto). Cells were then cultured for 24 h, and the medium collected in order to assay the amount of secreted angiotensinogen.

3. RESULTS

Bouhnik and colleagues [24] have demonstrated that the Reuber H35 hepatoma cell line produces angiotensinogen. According to their procedure, we

measured angiotensinogen in culture medium of H35 cells by RIA using antibody against rat angiotensinogen. The immunological identity of the material measured in the culture medium as rat angiotensinogen was confirmed by the fact that a series of dilutions of the medium gave a displacement curve parallel with the standard curve for purified rat angiotensinogen by RIA.

The angiotensinogen concentration in the culture medium of H35 cells increased linearly for at least 48 h under either dexamethasone-free or dexamethasone-supplemented (10^{-7} M) conditions (not shown). Fig.1 shows the amount of angiotensinogen secreted by H35 cells 24 h after the addition of several concentrations of dexamethasone. Angiotensinogen secretion by H35 cells was increased by the addition of dexamethasone; maximum response (4-fold) to dexamethasone was obtained at 10^{-7} M, half-maximal being at 10^{-8} M. Initial cell number (1.18×10^6/well) increased slightly to 1.32×10^6/well after 24 h culture in dexamethasone-free medium, and the addition of 10^{-7} M dexamethasone did not affect cell growth (1.29×10^6/well after 24 h culture).

Cytokines including IL-1, tumor necrosis factor, IL-6 and IFN-α have been identified as factors responsible for stimulating the hepatic production of acute-phase reactants [14–20]. In order to ascertain whether some of these cytokines have stimulatory activity on angiotensinogen production by H35 cells, cells were cultured for 24 h in the presence of dexamethasone (10^{-7} M) and several concentrations of IL-1α, IL-6 or IFN-α. Tumor necrosis factor could not be examined because of its cytotoxic activity on the tumor cells. As shown in fig.2, angiotensinogen secretion by H35 cells was significantly increased by IL-6, but not changed by IL-1α or IFN-α. Since the concentration of IL-1α or IFN-α would have been sufficient to stimulate the hepatic synthesis of other acute-phase reactants, such as α_1-acid glycoprotein by IL-1 in H35 cells [18] and T-kininogen by IFN-α in primary cultured rat hepatocytes [17], both cytokines seemed to be inactive with regard to angiotensinogen production by H35 cells.

Fig.3 shows the dose-response curve obtained after treatment of the cells with IL-6 for 24 h in the presence or absence of glucocorticoid. In the presence of 10^{-7} M dexamethasone, which produced a maximum response of H35 cells (fig.2),

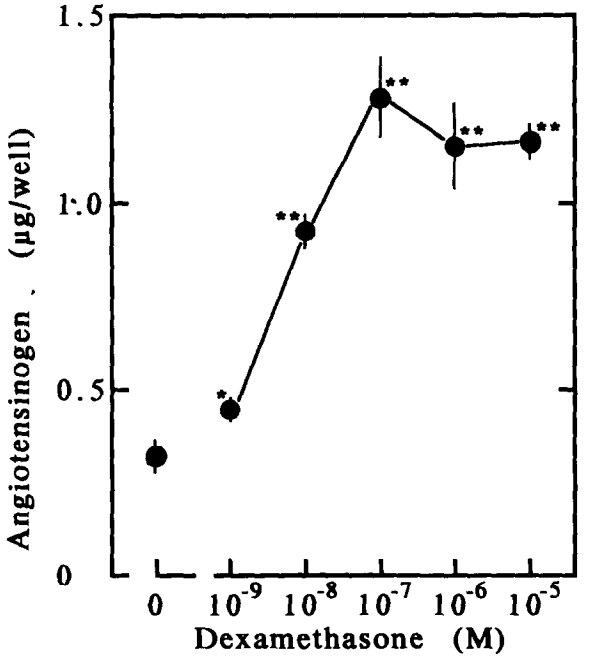

Fig.1. Secretion of angiotensinogen by H35 cells cultured with different concentrations of dexamethasone. H35 cells were cultured for 24 h in 2 ml of medium supplemented with different concentrations of dexamethasone, and angiotensinogen secreted into the medium was assayed. Results represent means ± SD for 4 cultures. Significantly different from the value without dexamethasone: $*p<0.01$, $**p<0.0001$.

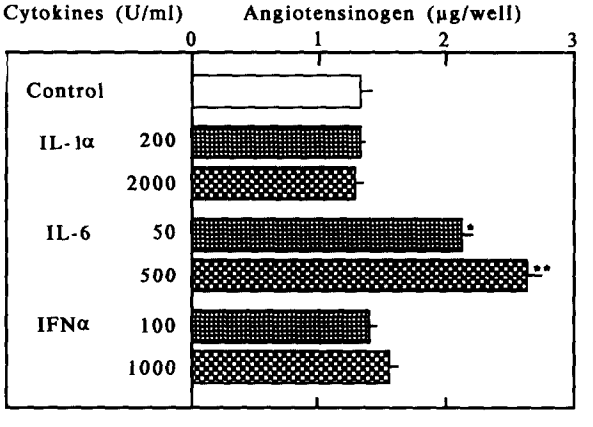

Fig.2. Effects of cytokines on the secretion of angiotensinogen by H35 cells. IL-1α, IL-6 and IFN-α were added to culture medium containing 10^{-7} M dexamethasone, and H35 cells were cultured for 24 h. Concentrations of cytokines are shown. Control represents a group cultured with dexamethasone alone. Results represent means ± SD for 4 cultures. Significantly different from the values of the control: $*p<0.01$, $**p<0.001$.

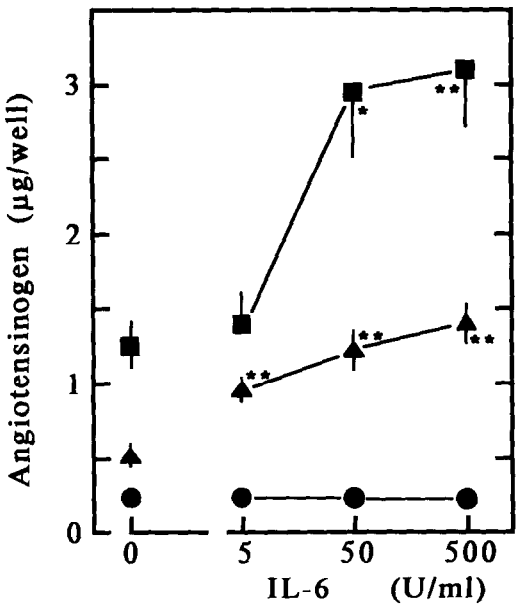

Fig.3. Dose response of IL-6-induced secretion of angiotensinogen by H35 cells cultured with different concentrations of dexamethasone. H35 cells were cultured in medium supplemented with several concentrations of IL-6 for 24 h without dexamethasone (●), with 10^{-8} M (▲) or 10^{-7} M dexamethasone (■). Results represent means ± SD for 4 cultures. Significantly different from the values without IL-6: $^{*}p<0.01$, $^{**}p<0.001$.

IL-6 at 50 U/ml further stimulated angiotensinogen secretion more than 2-fold as compared with dexamethasone alone. Dose-dependent stimulation of angiotensinogen secretion by IL-6 was also observed in the presence of 10^{-8} M dexamethasone. In contrast, IL-6 was entirely inactive under glucocorticoid-free conditions.

4. DISCUSSION

The liver has been shown to be the major source of plasma angiotensinogen and is therefore important in the regulation of circulating angiotensinogen levels [1]. Plasma levels of angiotensinogen are regulated by a variety of hormones, such as glucocorticoid, estrogen and thyroid hormones [1]. Treatment of rats with these hormones increases angiotensinogen mRNA levels in the liver and other tissues [30]. Bouhnik et al. [24] have demonstrated that the Reuber H35 rat hepatoma cell line synthesizes angiotensinogen. Chang and Perlman [4] have also shown that the angiotensinogen mRNA levels of H35 cells increase following treatment with dexamethasone, aldosterone or triiodothyronine. Thus, the H35 cell line is a well-defined one for studies on the regulation of angiotensinogen synthesis. This was further confirmed in the present study by measuring angiotensinogen secreted from H35 cells. Consistent with the results of studies on angiotensinogen mRNA levels, angiotensinogen secretion by H35 cells was increased 4-fold by 10^{-7} M dexamethasone, and the magnitude of the increase and half-maximal concentration of dexamethasone were comparable to those reported for the response of angiotensinogen mRNA [4].

Using this in vitro system, cytokines including IL-1, IL-6 and IFN-α were examined. Of these, only IL-6 had potent stimulatory activity; production of angiotensinogen maximally induced by 10^{-7} M dexamethasone was further stimulated by 50 U/ml IL-6. Interestingly, the presence of glucocorticoid was an absolute requirement for angiotensinogen induction by IL-6. In previous studies, we demonstrated that lipopolysaccharide-induced acute inflammation does not induce elevation of plasma angiotensinogen concentration in adrenalectomized or hypophysectomized rats [7,10]. Thus, the requirement for glucocorticoid of the effect of IL-6 observed in vitro is compatible with the findings regarding intact animals. It is well known that the synthesis of acute-phase reactants in the liver requires glucocorticoid [31]. For example, increases in β-fibrinogen or α_1-acid glycoprotein mRNA levels in rat hepatoma cells produced by IL-6 are potentiated by or absolutely dependent upon the presence of glucocorticoid [32]. In this respect, it appears that IL-6 stimulates the hepatic synthesis of angiotensinogen by a mechanism similar to that which regulates the synthesis of other acute-phase reactants.

IL-6 has recently been recognized as a major mediator of the acute-phase response [19,20], indicating that its action is not restricted to the immune system. The recent findings that HSF (IL-6) and IL-1 stimulate pituitary cells to release adrenocorticotropic hormone [33,34] suggest that molecules involved in the immune system can also control neuroendocrine functions. To our knowledge, the present study is the first to provide evidence that a mediator involved in immune func-

tion can modulate the function of the renin-angiotensin system. Many investigators have obtained data suggesting that, in addition to the renin concentration in plasma, the plasma level of angiotensinogen is a rate-limiting factor in the generation of angiotensin I and ultimately important in the regulation of blood pressure [1,3]. In fact, we have found that the level of angiotensin I generation is high in plasma obtained from rats with acute inflammation [35]. Therefore, the evidence provided here may also be further indicative of communication between the immune and endocrine systems.

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