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Mitogenic effects of coagulation factor XII and factor XIIa on HepG2 cells

KATHERINE T. SCHMEIDLER-SAPIRO*, OSCAR D. RATNOFF†, AND ERLINDA M. GORDON*‡

*Division of Hematology–Oncology, Childrens Hospital of Los Angeles, University of Southern California, Los Angeles, CA 90027; and †Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106

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ABSTRACT **The structure of coagulation factor XII (Hageman factor), inferred from its DNA sequence, includes two epidermal growth factor (EGF)-homologous domains in its amino-terminal region. This suggests that factor XII may exhibit EGF-like activities. Reciprocal antigenic cross-reactivity between factor XII and EGF was shown by exposing purified human factor XII or mouse EGF to anti-mouse EGF or anti-human factor XII. Western blot analysis showed that anti-mouse EGF recognized intact factor XII at 80 kDa. Together, these results suggest that the EGF-homologous domains are accessible for anti-EGF binding in native factor XII. To determine whether factor XII has mitogenic activity, HepG2 or L cells (10^4 cells per well) were grown in serum-free medium in the presence or absence of factor XII or kaolin-activated factor XII (factor XIIa). Both factors XII and XIIa (6.0 μg/ml) enhanced cell proliferation by ≈2-fold ($P < 0.001$ and $P < 0.005$, respectively). In contrast, L cells, which are not EGF target cells, were not affected by either factor XII or factor XIIa. Various doses of factor XII enhanced cell proliferation, [^{3}H]thymidine incorporation, and [^{3}H]leucine incorporation in HepG2 cells cultured under the same conditions. These data indicate that factor XII, like EGF, is a mitogen for HepG2 cells and suggest a possible autocrine role in the liver.**

Factor XII is a procoagulant, found in normal plasma, that participates directly or indirectly in activation of the intrinsic clotting pathway, factor VII, fibrinolysis, and the renin–angiotensin and kallikrein–kinin systems (1–3). The structure of factor XII, inferred from its DNA and amino acid sequences, includes two epidermal growth factor (EGF)-homologous domains in the amino-terminal region (4–7). This suggests that factor XII may mimic EGF and act as a growth factor. Hepatocytes and hepatoma cells are target cells for EGF and hepatocytes synthesize factor XII, raising the possibility that factor XII may act as an autocrine or paracrine hepatocyte growth factor, perhaps in a feedback process, to regulate liver growth or productive activity under physiologic conditions.

EGF is a well-characterized mitogen that stimulates the proliferation of numerous cell types *in vitro* and of epithelial cells *in vivo*. The EGF receptor is detectable on a large variety of cell types, mostly epithelial. Available evidence indicates that the EGF receptor mediates the biological signals of EGF and possibly those of two EGF-like growth factors—namely, transforming growth factor α and the vaccinia virus growth factor (8), which have amino acid sequences homologous to EGF (9). Since the putative tertiary structure of factor XII includes domains that appear to be homologous to EGF (4–7), we examined whether factor XII might have growth factor activity as well.

Growth factors can elicit numerous responses in target cells. Among these are (*i*) early cellular responses, such as increased protein synthesis; (*ii*) later cellular responses, such as increased DNA synthesis; and (*iii*) the definitive cellular response to a mitogen, an increased mitotic rate, and, therefore, increased cell numbers. If factor XII were to elicit any or all of these responses in hepatocytes, it would suggest that this protein might participate in an autocrine feedback system.

MATERIALS AND METHODS

Materials. HepG2 cells (a human hepatoma cell line) and L cells were supplied by American Type Culture Collection. Mouse EGF was supplied by Collaborative Research. Human serum albumin (99% pure) was a gift from Armour Pharmaceutical. Purified human factor XII (specific activity, 123 units per mg of protein), mouse EGF, polyclonal rabbit anti-mouse EGF IgG, and rabbit anti-human EGF antiserum were supplied by Collaborative Research.

Purification of Factor XII. Purified rat factor XII (specific activity, 22.3 units per mg of protein) was prepared from citrated pooled plasma of Sprague–Dawley rats (Pel-Freez Biologicals) by sequential chromatography as described (10). This factor XII was devoid of detectable amounts of other clotting factors and formed a single 80-kDa band on SDS/PAGE in both reduced and nonreduced samples. Activated factor XII (factor XIIa) was prepared as described (11, 12). This preparation, which we have called factor XIIa in these experiments, consisted of factor XII activated by kaolin but not purified further. Activation of factor XII is a two-step process. The first proteolytic event splits factor XII into two fragments linked by a disulfide bridge, a split that activates factor XII. A second proteolytic step degrades the larger fragment further into a carboxyl-terminal fragment with coagulant activity and a larger amino-terminal fragment, which retains the fibronectin and EGF-like domains. Our activated factor XII preparation is probably a mixture of these two forms.

Factor XII–EGF Antigenic Cross-Reactivity by Western Blotting. Immunoreactive factor XII and EGF were prepared for Western immunoblotting as described (13). EGF and rabbit antisera directed against mouse EGF (anti-EGF) were supplied by Collaborative Research. Monospecific IgG directed against human factor XII was prepared in New Zealand albino rabbits as described (14–16). After electrophoresis and transfer to nitrocellulose, blots were developed by the Bio-Rad Immun-Blot assay procedure.

Culture of HepG2 and L Cells in Serum-Free Medium. Cells (10^4 or 10^5 cells per well) were plated in 24-well plates (each 16 mm diameter) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and grown to confluence (1 week). Medium was then replaced with serum-free medium

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Abbreviations: EGF, epidermal growth factor; TCA, trichloroacetic acid.

‡To whom reprint requests should be addressed at: Childrens Hospital of Los Angeles, Division of Hematology–Oncology, 4650 Sunset Boulevard, Building C, Los Angeles, CA 90027.

(1 ml per well) (17, 18) for 48 hr before treatment with specific agents. The serum-free medium was an equal mixture of Williams E medium and Ham's F-12 medium (GIBCO), supplemented with 0.25% sodium bicarbonate, Hepes (15 mmol/liter), penicillin (500 units/ml), streptomycin (500 μg/ml), ethanolamine (65.5 μmol/liter), transferrin (100 μg/ml), insulin (0.6 μg/ml), hydrocortisone (1.0 nmol/liter), trace elements, linoleic acid (7.18 μmol/liter) linked to 0.08% fatty acid-free bovine serum albumin, glucose (7.0 mmol/liter), sodium pyruvate (0.4 mmol/liter), arginine (0.6 mmol/liter), and ascorbic acid (1.0 mmol/liter).

Cell Counting. To monitor cell growth, triplicate wells were assessed. After trypsin treatment, an aliquot of suspended cells from each well was transferred into a standard hemocytometer and the cells were counted (19).

[^{3}H]Thymidine Incorporation Assay. [^{3}H]Thymidine incorporation into macromolecules was measured in untreated (control) cells and in cells treated with either EGF or factor XII. Briefly, the cells were incubated with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) (NEN) for 2 hr at 37°C. The cells were then gently washed with ice-cold sodium phosphate, 0.15 ionic strength (pH 7.4), and dispersed with trypsin-EDTA. The cells were homogenized and a denaturing solution [2 drops of 5 M NaOH and 5 ml of 10% trichloroacetic acid (TCA)] was added. Samples were then poured on glass filters wetted with ice-cold 2% TCA and vacuum-dried. The glass filters were then washed with ice-cold 2% TCA and dried in an oven at 80°C for 1 hr. Radioactivity in the dried filters was counted by using a Nuclear-Chicago γ and scintillation counter. [^{3}H]Thymidine incorporation into cells was expressed as absolute radioactivity (cpm per well).

[^{3}H]Leucine Incorporation Assay. [^{3}H]Leucine incorporation into macromolecules was measured by TCA precipitation of radiolabeled peptide products in untreated (control) cells and in cells treated with either EGF or factor XII. After exposure to the respective agent, cells were incubated for 1 hr in the presence of [^{3}H]leucine. At the end of the labeling period, the medium was removed, and the cells were washed with fresh medium to remove free label. Cells were solubilized in 1.0 ml of 0.2 M NaOH containing 500 μg of bovine serum albumin per ml as a carrier, the plate was rinsed with 1.0 ml of the same NaOH solution, and macromolecules in the pooled sample were precipitated by the addition of 6.0 ml of ice-cold 10% TCA for 10 min on ice. Samples were heated to 90°C for 5 min to hydrolyze aminoacylated tRNA and then washed several times with ice-cold 5% TCA. Precipitates were collected by filtration. Total ^{3}H incorporated into hot TCA precipitable polypeptides was measured by liquid scintillation counting.

The effect of rabbit anti-EGF antiserum on the activation or action of factor XII was measured by incubating 0.1 ml of purified human factor XII (0.02 unit/ml in 0.25% bovine serum albumin in barbital/saline buffer) with 0.1 ml of either crude IgG rabbit anti-EGF antiserum or normal rabbit serum, or bovine serum albumin (3.6 mg of protein per ml of barbital/saline buffer) for 10 min at 37°C in polystyrene tubes (12 × 75 mm). Ellagic acid (0.1 ml; 2.5×10^{-5} M in barbital/saline buffer) was added to each tube and incubation was continued for 1 hr. Then 1.0 ml of 5×10^{-4} M S2302 was added to each tube, and the incubation was continued for 1 hr. The reaction was stopped by addition of 0.3 ml of glacial acetic acid, and the amount of nitroaniline released was measured at 405 nm, using suitable blanks.

Statistical Analysis. The significance of differences in cell number and [^{3}H]thymidine or [^{3}H]leucine uptake among EGF-, factor XII-, or factor XIIa-treated and untreated groups was evaluated by analysis of variance (20).

RESULTS

Factor XII and EGF Antigenic Comparison. In an immunoblot assay (13), anti-mouse EGF reacted with two preparations of purified human factor XII (specific activity, 22.3 and 123 units per mg of protein, respectively) forming clear precipitin lines. Using standard Ouchterlony techniques, this anti-EGF preparation readily precipitates mouse EGF. Conversely, anti-rat factor XII IgG reacted with purified EGF, suggesting that this antibody recognizes EGF domains in factor XII. These data suggested that EGF-homologous regions are accessible for specific binding in intact factor XII, as well as in the amino-terminal fragment liberated during activation of this clotting factor. To demonstrate more conclusively that these EGF-like domains are accessible in intact polypeptides, not just contaminating fragments, Western blots were prepared. This analysis showed that anti-mouse EGF recognized intact human factor XII at 80 kDa (Fig. 1). These results strongly suggest that the EGF-homologous domains are accessible for anti-EGF binding in native factor XII.

Mitogenicity of Factor XII and Factor XIIa on HepG2 and L Cells. HepG2 cells or L cells were prepared and treated as described above. Factor XII was activated by treatment with kaolin and soybean phosphatides as reported (11, 12). Increased cell counts were seen after addition of factor XII (6 μg/ml) ($P < 0.001$) or factor XIIa (6 μg/ml) ($P < 0.005$) (Table 1). In contrast, L cells, which are not EGF target cells, were not affected by either factor XII or factor XIIa (Table 1). As expected, EGF (500 ng/ml) treatment resulted in increased cell numbers in hepatoma cells ($P < 0.01$) but not in L cells (Table 1).

Dose-Dependent Mitogenic Effects of Intact Factor XII on HepG2 Cells. HepG2 cells were inoculated into serum-free medium at 10^5 cells per well. To assess cell growth, overnight cultures were incubated for 24 hr in the presence or absence of factor XII (Fig. 2). Factor XII enhanced cell proliferation in a dose-dependent manner ($P < 0.001$) compared to cells incubated in the same medium only lacking factor XII (Fig. 2*A*). Hepatoma cells typically form many clumps after trypsin treatment, accounting to some degree for the variance among replicates. Factor XII-treated hepatoma cells showed this tendency more markedly than untreated cells. In all cases, a clump was counted as a single unit. Therefore, any error introduced by this method would have resulted in a relative undercounting of factor XII-treated cells, implying that the actual effect may have been higher than stated in our results.

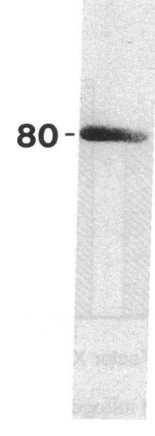

FIG. 1. Factor XII–EGF cross-reactivity by Western blot analysis. Purified human factor XII immunoprecipitated with anti-mouse EGF at 80 kDa, indicating that EGF-homologous domains in intact factor XII are accessible for anti-EGF binding.

Table 1. Effect of coagulation factors XII and XIIa on cell proliferation

Treatment group	Cell count per well, $\times 10^{-4}$	
	HepG2 cells	L cells
Control (untreated)	1.2 ± 0.1	5.9 ± 0.7
EGF (500 ng/ml)	2.2 ± 0.4 ($P < 0.01$)	5.3 ± 0.6 (NS)
Factor XII (6 μg/ml)	2.2 ± 0.1 ($P < 0.001$)	5.7 ± 0.3 (NS)
Factor XIIa (6 μg/ml) + kaolin/phospholipid (0.5 mg/ml)	2.2 ± 0.2 ($P < 0.005$)	5.7 ± 0.4 (NS)
Kaolin/phospholipid (0.5 mg/ml)	1.2 ± 0.3 (NS)	4.7 ± 1.2 (NS)

Results are expressed as arithmetic means ± SD ($n = 4$ per group). P values were evaluated by the two-tailed t test. NS, not significant. The added kaolin/phospholipid mixture was not removed from the cultures during the incubation period.

[^{3}H]Thymidine Incorporation into DNA. An alternative method to assess mitogenicity is to measure rates of [^{3}H]thymidine incorporation into DNA. HepG2 cultures (original inoculum, 10^5 cells per well) were grown to confluence (≈5 days). Cells were washed with serum-free medium and then reincubated in fresh serum-free medium. Overnight cultures were then incubated in the presence or absence of factors, as indicated, for 24 hr before [^{3}H]thymidine was added. Factor XII enhanced [^{3}H]thymidine incorporation by HepG2 cells in a dose-dependent manner, measured after a 2-hr pulse (Fig. 2*B*). In contrast, [^{3}H]thymidine incorporation by L cells was not significantly affected by factor XII treatment. This is in agreement with the cell count data described above.

[^{3}H]Leucine Incorporation into Protein. In separate experiments, cells were treated as described above. After a 2-hr pulse with [^{3}H]leucine, cells treated with factor XII showed increased leucine incorporation into macromolecules (Fig. 2*C*).

Effect of Anti-EGF on the Activation and Action of Factor XII. Under the conditions tested, a crude IgG directed against human EGF did not alter the activation of factor XII by ellagic acid nor did it inhibit the amidolytic properties of activated factor XII (data not shown).

DISCUSSION

In this study, factors XII and XIIa enhanced cell proliferation and thymidine and leucine incorporation in HepG2 cells. Since EGF-homologous domains are found in the amino-terminal fragment of factor XII, these data suggest that these mitogenic responses represent EGF-like activity of factor XII. In support of this hypothesis, EGF induced a similar response in HepG2 cells. In contrast, neither factor XII nor EGF induced cell proliferation in L cells, nontargets of EGF. As our data do not directly show that factor XII binds to the EGF receptor, further experiments will be necessary to clarify this point. Alternatively, preparations of factor XII may have mitogenic properties independent of its EGF-homologous domains. Anti-EGF antibody has no inhibitory effects on the activation of factor XII (a function of its amino-terminal region) or on the amidolytic activity of factor XII (a property belonging to its carboxyl-terminal region).

Since factor XII is synthesized by hepatocytes, these data indicate that factor XII may act as an autocrine or paracrine hormone, or both. Tissue growth factors that may be involved in liver regeneration have recently become the object of study (21–26). Transforming growth factor α bears structural homology to EGF and appears to act at the EGF receptor (27). Serum EGF levels do not appear to increase after partial hepatectomy, suggesting that transforming growth factor α and possibly other mitogenic factors, rather than EGF itself, may be primarily responsible for regulating liver cell proliferation during regeneration (21). Several other hepatic mitogens have been identified, including basic fibroblast growth factor (24), heparin-binding growth factor type 1 or acidic fibroblast growth factor (25), and "hepatotropin" (26). These earlier investigations all focused on hepatic regeneration. As demonstrated here, factor XII may also be a hepatic mitogen and thus might participate in the complex regulation of liver regeneration.

Perhaps, under ordinary circumstances, factor XII growth factor activity does not result in mitogenicity *per se*; pre-

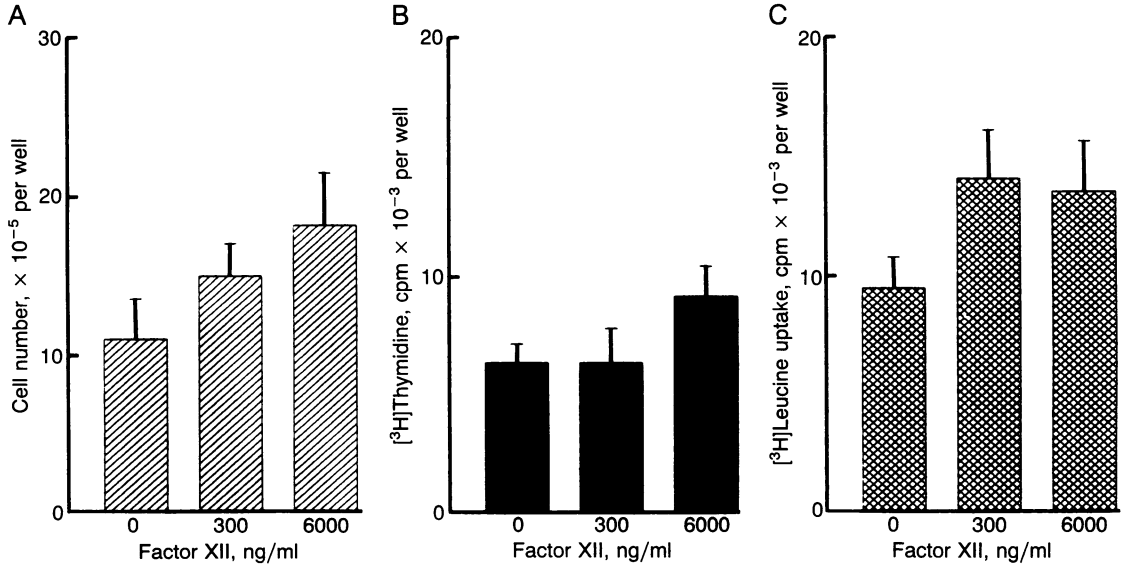

FIG. 2. Dose-dependent mitogenic effects of factor XII on HepG2 cells. HepG2 cells were plated at an initial plating density of 10^5 cells per well. Cultures were grown to confluence for ≈5 days. Cells were washed with serum-free medium and then resuspended in fresh serum-free medium. Factor XII was added to overnight cultures at the concentrations indicated, and cells were further incubated for 1 day. [^{3}H]Thymidine or [^{3}H]leucine was added for a 2-hr pulse period. Then cells were washed extensively with serum-free medium and treated with trypsin. (*A*) Cell counts using a hemocytometer are expressed as number of cells per well. (*B*) Incorporation of [^{3}H]thymidine in cells is expressed as cpm per well. (*C*) Incorporation of [^{3}H]leucine in cells is expressed as cpm per well.

sumably, other mitogenic processes may be more important. The mitogenic response is itself complex. To induce cell growth, a mitogen must also stimulate synthesis of both specific and nonspecific RNA and protein products. Increased rates of RNA and protein synthesis in response to increased levels of activated factor XII (or factor XIIa) could provide an organism with an appropriate response to trauma associated with bleeding as well as local injury to the liver itself. The same reaction that activates factor XII for coagulation may liberate the amino terminus with its associated EGF domains. These may then enhance protein production in the liver. Whether this is product specific for coagulation factors or nonspecific enhancement of liver function is one interesting question raised by this work. Either way could be envisioned as physiologically important, since an organism under severe stress requires a pleiotropic response, not a highly specific one. Thus, this growth factor-like activity of factor XII may have a role in elucidating normal liver function as well as hepatic response to systemic trauma.

If factor XII mitogenic activity derives from its homology with EGF, then this activity may not be limited to cells of hepatic origin. Endothelium is one of the many targets of EGF and may thus be a target of factor XII as well. There are also pathological implications. Abnormally high levels of factor XII have been associated with coronary heart disease (3). Increased hepatic synthesis of clotting factors has been implicated in the pathogenesis of thrombotic disorders. Since factor XII can stimulate hepatic protein synthesis, it may stimulate its own production in a positive feedback loop. This may be one mechanism through which abnormally high levels of factor XII, and possibly other coagulation factors, are attained.

Elucidation of the factor XII mitogenic potential will add an exciting role for factor XII, which bears sequence homologies with EGF and fibronectin.

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