Abstract:
We have previously demonstrated that only when serum was included in the culture medium, heparin-binding fibroblast growth factor-1 (FGF-1) was a potent mitogen for rat hepatocytes in primary culture. Here we have defined the constituents of serum that is important for the mitogenic activity of FGF-1. Addition of dialyzed serum to the basal medium supported about half of the FGF-1 activity compared to whole fetal bovine serum (FBS). Further addition of ascorbic acid 2-phosphate to this medium reconstituted the full activity of FGF-1. However, ascorbic acid 2-phosphate alone could only partially support the FGF-1 mitogenicity. These findings indicate that dialyzed-FBS and ascorbic acid 2-phosphate additively or synergistically support the mitogenic activity of FGF-1. These effects of dialyzed FBS and ascorbic acid 2-phosphate were observed regardless of the basal medium used, i.e., Williams' medium E or MCDB 107 medium. Thus, these results suggest that the high molecular-weight factor(s) and ascorbic acid-like compound(s) are the important constituents of serum that support the FGF-1 mitogenic activity.

Key words: Hepatocytes, FGF-1, Ascorbic acid, Serum

Introduction
In the initial stage of liver regeneration, the level of heparin-binding fibroblast growth factor-1 (FGF-1) transcript in liver remnant is promptly increased\(^1,2\). From the observation, it has been suggested that FGF-1 is a significant factor for the growth of liver parenchymal cells in vivo. Indeed, in the primary culture of rat hepatocytes, the mitogenic activity of FGF-1 is comparable with those of the well-described hepatocyte mitogens, such as epidermal growth factor (EGF) or hepatocyte growth factor (HGF)\(^3-6\). Interestingly, the maximum activity of FGF-1 in rat hepatocytes required the addition of fetal bovine serum (FBS) as described previously\(^3\). Since the mitogenic activity of EGF did not depend on FBS\(^5\), it is suggested that FBS specifically supports the mitogenic activity of FGF-1. To clarify the crucial roles of FBS in the FGF-1 mitogenicity, we examined the effect of dialyzed-FBS. Although the dialyzed-FBS was not able to sustain the full activity of FGF-1, the combination usage with ascorbic acid 2-phosphate, which is a stable derivative of L-ascorbic acid\(^7\), exerted the comparable activity with whole FBS.
Materials and Methods

Hepatocytes were isolated from adult female Wistar rats (7-9 weeks old) by two-step collagenase perfusion in situ as reported previously\(^3\). More than 95% of cells were morphologically identified as hepatocytes. Cells were plated at 2 x 10^4 cells/cm\(^2\) in the Type I-collagen coated 24 well plate (Celltight C-I Plate, Sumitomo Bakelite, Tokyo) containing 0.5 ml of Williams' medium E with 10% FBS (Irvine Scientific, CA) and 10^-7 M dexamethasone. The cells were cultured under the atmosphere of 5% CO\(_2\), 95% air at 37°C. After 2 h of plating, the medium was replaced with 0.5 ml of MCDB 107 Medium (MCDB 107 (Kyokuto Pharmacerutical, Tokyo) containing 1 mg/ml bovine serum albumin, 4 μg/ml oleic acid, 5 μM dithiothreitol, 5 μg/ml ethanolamine, 10^-7 M insulin), or WE Medium (Williams' medium E (ICN Biochemicals, CA) supplemented with 10^-7 M dexamethasone, and 10^-7 M insulin). Simultaneously, FBS (10%), dialyzed-FBS (10%) and/or ascorbic acid 2-phosphate (2 mM) were added to the culture in the presence or absence of 10 ng/ml human recombinant FGF-1\(^9\) supplemented with 5 μg/ml heparin (Sigma Chemicals, MO), and then the cells were further incubated. Dialyzed-FBS was prepared from the same lot of FBS (50 ml) by dialysis (molecular weight 8-kDa cut off) against 150 mM NaCl (4 litter for 12 h, 4 times) at 4°C. Ascorbic acid 2-phosphate and hypoxanthine were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma Chemicals (MO), respectively.

Total DNA synthesis was determined by the incorporation of \[^3\text{H}\] thymidine (Moravek Biochemical, CA) into DNA. For the MCDB 107 Medium, the cells were labeled with \[^3\text{H}\] thymidine (9.62 kBq/ml, 5.22 GBq/mmol) from 48 to 72 h after the cell seeding according to the method of Kan et al.\(^1\). For WE Medium, the cells were incubated with \[^3\text{H}\] thymidine (9.25 kBq/ml, 6.18 GBq/mmol) from 30 to 54 h after the cell seeding as described previously\(^9,10-12\). Labeling was terminated by washing the cells with cold phosphate buffered saline (PBS), and the radioactivity was determined by liquid scintillation counter as described\(^3\).

Results

Consistent with our previous report\(^2\), the FGF-1 mitogenic activity in rat hepatocytes was potentiated about 5 fold by the supplementation of 10% FBS in MCDB 107 Medium (Fig. 1). To address the effective component(s) in FBS for the enhancement of FGF-1 mitogenicity, we used the dialyzed-FBS which was obtained from same batch of the FBS by dialysis (molecular weight 8-kDa cut off). Although the dialyzed-FBS (10%) also supported the mitogenic activity of FGF-1, the enhanced level was about 3 times over the serum-free condition (Fig. 1). This supporting activity was not due to the mitogenic activity by dialyzed-FBS itself (Fig. 1), and the activity was decreased by the addition of higher concentration of dialyzed-FBS (20%; data not shown). These results indicate that the dialyzed-FBS retains a part of the component(s) which supports the FGF-1 mitogenic activity.

The findings described above also suggest that the supporting-activity by whole FBS was partly provided by low molecular-weight compo-
FGF-1 Mitogenicity in Rat Hepatocytes

nent(s) in FBS. Then, we examined the effect of ascorbic acid 2-phosphate, because ascorbic acid not only acts as an anti-oxidative agent but also promotes many biological activities in cultured cells; it has been reported that ascorbic acid activated the metabolism of extracellular matrix components\textsuperscript{13). Interestingly, under serum free condition, the supplementation of ascorbic acid 2-phosphate potentiated cellular DNA synthesis induced by FGF-1, and the potentiation was about 2 fold (Fig. 1). The mitogenic activity of ascorbic acid 2-phosphate itself was negligible. Furthermore, the effect of hypoxanthine was examined, because it was reported that the supplementation of hypoxanthine with dialyzed-FBS was required for the maximal growth stimulation of endothelial cells by FGF-1\textsuperscript{14,15). However, the addition of hypoxanthine (50 $\mu$M) to the culture medium in rat hepatocytes could not enhance the FGF-1 mitogenic activity (data not shown). These results indicate that ascorbic acid-2 phosphate is a supporting factor for FGF-1 mitogenicity in rat hepatocytes.

To test the possibility that the function of the dialyzable fraction of FBS can be sustained by ascorbic acid 2-phosphate, the combined effect of dialyzed-FBS and ascorbic acid 2-phosphate on the FGF-1 mitogenicity was examined. The supporting activity for FGF-1 mitogenicity was obviously increased by the combined addition, and the enhanced level was comparable with that by whole FBS (Fig. 1). These results indicate that the combined usage of dialyzed-FBS and ascorbic acid 2-phosphate completely reconstitutes the supporting activity of whole FBS for the FGF-1 mitogeneity.

For WE Medium, the effects of dialyzed-FBS and ascorbic acid 2-phosphate on the FGF-1 mitogenicity for rat hepatocytes were examined, since the potentiation of FGF-1 activity by whole FBS was observed in this culture condition, as shown in Fig. 2. The addition of dialyzed-FBS or ascorbic acid 2-phosphate induced the enhancement of FGF-1 mitogenicity as well as those observed in MCDB 107 Medium, and the full-supporting activity for the FGF-1 mitogenicity was obtained by the combined addition of both (Fig. 2). These data indicate that the supporting effect of dialyzed-

FBS and ascorbic acid 2-phosphate on the FGF-1 mitogenicity is a prevalent aspect irrespective of the basal medium.

To assess the function of ascorbic acid 2-phosphate as anti-oxidative agent, we examined the effect of dithiothreitol on the FGF-1 activity using WE Medium as a basal medium. As shown in Fig. 3, ascorbic acid 2-phosphate supported the FGF-1 mitogenicity in dose-dependent manner. In a similar manner, the lower-dose of dithiothreitol (10 $\mu$M) enhanced the FGF-1 mitogenicity to the comparable level with that of ascorbic acid 2-phosphate, whereas the addition of higher-dose (more than 100 $\mu$M) inhibited it (Fig. 3). In addition, the combined usage of dithiothreitol (1-100 $\mu$M) and dialyzed-FBS (10%) sustained the full-mitogenic activity for FGF-1 (data not shown). These results indicate that ascorbic acid 2-phosphate supports the FGF-1 activity through the similar function(s) with dithiothreitol.

Discussion

In the present work, we have demonstrated

![Graph](image-url)
that the dialyzed-FBS and ascorbic acid 2-phosphate have a supporting activity for FGF-1 mitogenicity in rat hepatocytes, and that the combined usage of both restore the supporting activity comparable with that of whole FBS. These findings suggest that the component(s) in dialyzed-FBS and the ascorbic acid-like compound(s) additively or synergistically sustain the mitogenic activity of FGF-1.

Dialyzed-FBS itself did not act as a mitogen for rat hepatocytes, and it supported the FGF-1 mitogenicity, suggesting that the high molecular-weight factor(s) in serum include a part of effective component(s) for FGF-1 activity. Since the heat-treated serum (95°C, 10 min) has the full-supporting activity for FGF-1 mitogenicity in rat hepatocytes (data not shown), it is suggested that the high molecular-weight factor(s) may be heat-stable compound(s).

By the treatment with ascorbic acid 2-phosphate, it has been reported that the deposition of acidic glycosaminoglycans, especially heparan sulfate, is increased in fibroblasts. Furthermore, in the several types of cells, the highly-sulfated heparan sulfate is essential for the FGF mitogenicity. These results raise a possibility that the contribution of ascorbic acid 2-phosphate is directly involved in the functions of heparan sulfate for FGF-1 mitogenicity. However, it is hard to conclude that ascorbic acid 2-phosphate sustains the FGF-1 mitogenicity through the increased deposition of heparan sulfate by the cells, because the roles of heparan sulfate in the FGF mitogenicity can be replaced by the supplement of heparin, and heparin was included in the culture medium used in the present experiments.

Interestingly, we demonstrate that not only ascorbic acid 2-phosphate but also dithiothreitol enhance the mitogenicity of FGF-1 in serum-free medium, whereas these increased levels were much less than that of whole FBS. The combined addition of dithiothreitol and dialyzed-FBS supported the full-mitogenicity of FGF-1, as well as that of ascorbic acid 2-phosphate and dialyzed-FBS added to the serum-free culture medium. From these findings, it is suggested that a role of ascorbic acid 2-phosphate in the enhancement of FGF-1 activity involves the anti-oxidative action. However, since dithiothreitol was already supplemented in MCDB 107 Medium and the full-mitogenic activity of FGF-1 was not induced by the addition of dialyzed-FBS only, it is also anticipated that another function of ascorbic acid 2-phosphate contributes to sustain the FGF-1 mitogenicity. Thus, whereas the function of ascorbic acid 2-phosphate in FGF-1 mitogenicity has not been completely understood, it is concluded at least that the anti-oxidative compound is significantly effective for the FGF-1 mitogenicity, as demonstrated by Engleka and Maciag.

Herein, we described that the mitogenic activity of FGF-1 in rat hepatocytes was sustained by the components of serum which comprises the high molecular-weight component(s) and the ascorbic acid-like compound(s). While the detailed investigation about these components is necessary, the culture system described here would be a useful tool for the analysis for mechanisms of FGF-1 mitogenicity in rat hepatocytes.
FGF-1 Mitogenicity in Rat Hepatocytes

Acknowledgments

We thank to Takehito Uruno, Isami Tsuboi, Naoto Okamoto, Nobumitsu Koga, and Youji Mitsui for their helpful discussions and supports.

References


(Accepted 8 December 1997)