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Stimulation of liver functions in hierarchical co-culture of bone marrow cells and hepatocytes

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Abstract

A hierarchial co-culture, in which rat hepatocytes and non-parenchymal liver cells (NPLCs) were separated by a collagen layer and which was designed to mimic the *in vivo* microenvironment, was carried out with the aim of developing a module for bio-artificial liver support. Compared with a monolayer co-culture and hepatocytes cultured alone in a monolayer, higher urea synthesis activity was maintained for 6 d in the hierarchical co-culture. When a rat hepatoma cell line H4-II-E-C3, which retains the induction of tyrosine aminotransferase (TAT), was co-cultured in a monolayer with NPLCs, dose-dependent stimulation of TAT induction was observed. In a hierarchical co-culture, NPLCs further stimulated TAT induction in H4-II-E-C3 cells. Since peritoneal macrophages could stimulate TAT induction in hepatocytes in both monolayer and hierarchical co-cultures, bone marrow cells, which can proliferate and differentiate into macrophages *in vitro*, were investigated as a possible substitute for NPLCs. Bone marrow cells isolated from rat femurs were cultivated in the presence of IL-3 and macrophage colony-stimulating factor (M-CSF), and co-cultured with hepatocytes. Urea synthesis and TAT induction of hepatocytes were stimulated in the co-culture. The co-culture of bone marrow and H4-II-E-C3 cells, both of which have proliferation ability *in vitro*, was also shown to be effective in stimulating liver functions. The hierarchical configuration, in which two cell types can communicate with the soluble factor(s) through a collagen layer, was found to be more effective than a monolayer in long-term co-culture.

Abbreviations: BAL – bioartificial liver support system; NPLC – non-parenchymal liver cells; TAT – tyrosine aminotransferase; M-CSF – macrophage colony-stimulating factor

Introduction

Many attempts have been made to improve the survival rate of patients with hepatic failure by means of extacorporeal liver support systems, such as plasma exchange, hemodialysis, and charcoal hemoperfusion. However, apart from liver transplantation, there are few effective treatments leading to complete recovery. Even in the case of transplantation, because of a severe worldwide shortage of donor organs, many patients die before the appearance of a suitable donor. Development of an effective artificial liver support system that can be used as a bridge to transplantation for patients with chronic liver disease, as well as for those with acute hepatic failure, is thus urgently needed.

Recent developments in cell culture technology have made it possible for isolated hepatocytes to survive and maintain their functions *in vitro* over a long period. Several types of bioartificial liver support systems (BALs) employing cultured hepatocytes have been proposed (Nyberg et al., 1993, Rozga et al., 1994) because they have advantages over non-biological sup-

port systems, such as being able to perform synthetic functions. Hollow fiber bioreactors containing porcine hepatocytes (Demetriou et al., 1995, Rozga et al., 1994) or a human cell line (Sussman et al., 1994) have been used clinically in the United States to treat several patiens with severe hepatic failure. However, extensive improvements are still necessary to obtain an effective BAL system in terms of a higher patient survival rate and lower costs.

We have been investigating the use of non-parenchymal liver cells (NPLCs) as well as hepatocytes with the aim of constructing a BAL system that mimics the *in vivo* environment to sustain liver functions *in vitro*. Among our results, we found that a monolayer co-culture of hepatocytes and NPLCs stimulated the induction of tyrosine aminotransferase (TAT), which is a hepatocyte-specific function (Yagi et al., 1992); microencapsulated heterospheroids consisting of hepatocytes and NPLCs maintained albumin secretion and TAT induction longer than a spheroids consisting only of hepatocytes (Yagi et al., 1993); a hierarchical co-culture in which hepatocytes and NPLCs were separated by a collagen layer also exhibited a similar stimulatory effect (Yagi et al., 1995). In the latter configuration, a soluble factor(s) was able to diffuse from the NPLCs at the bottom of the dish through the collagen layer and reach the hepatocytes without dispersing into the medium. Among the NPLCs, Kupffer cells were found to be responsible for this effect. However, collecting a sufficient number of Kupffer cells for the construction of a BAL system presents a serious difficulty, because such cells constitute only 10% of the total liver cell population. According to the mononuclear phagocyte system, most macrophages are postulated to be derived from monocytes, which are differentiated via promonocytes from monoblasts originating in bone marrow (Furth et al., 1972). The precursors migrate to various tissues and differentiate into resident cells, such as Kupffer cells, and alveolar and peritoneal macrophages. An alternative mechanism, in which macrophages are differentiated directly from their hematopoietic precursor cells without passing through promonocyte or monocyte stages, has also been proposed (Takahashi et al., 1989). The proliferation ability of terminally differentiated macrophages appears to be poor, while the precursor cells in bone marrow proliferate extensively *in vitro* in the presence of IL-3 (Roberts et al., 1988; Whetton et al., 1983; Williams et al., 1990) and differentiate into macrophages with macrophage colony-stimulating factor (M-CSF) (Furth, 1989; Nicola et al., 1994). In the present study, macrophages differentiated from bone marrow cells *in vitro* were examined with respect to their effectiveness in a hierarchical co-culture with hepatocytes.

Materials and methods

Media

Basal media used for liver cells and the rat hepatoma cell line H4-II-E-C3 were Williams E medium (WE, ICN Biochemicals, Costa Mesa CA, USA) and Dulbecco's modified eagle medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan), respectively. Bone marrow cells, the L 929 fibroblastic cell line, WEHI-3 myelomonocytic leukemia cell line, and J774A.1 macrophage-like cell line were cultivated in RPMI1640 (Nissui Pharmaceutical) with appropriate concentrations of fetal bovine serum (FBS, ICN Biochemicals); 2.5% for L 929, 1.5% for WEHI-3, and 5% for J774A.1. The medium used for liver cell isolation was WE supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml fungisone, 1 nM insulin, and 1 nM dexamethasone (Medium A).

Cell isolation

Liver cells were isolated from male Sprague Dawley (SD) rats weighing 250–300 g by perfusion of the liver with 0.05% collagenase (182 units/mg, from *Clostridium histolyticum*; Wako Pure Chemicals, Osaka, Japan) by the method of Seglen (Seglen, 1976). The total liver cell suspension was centrifuged at $50 \times g$ for 30 s. The pellet was used as the hepatocyte fraction after further purification by two centrifugations at $50 \times g$ for 30 s. The isolated hepatocytes were suspended at 5×10^5 cells/ml in Medium A. The supernatant of the first centrifugation was centrifuged at $150 \times g$ for 90 s and the resulting pellet was used as the non-parenchymal liver cell (NPLC) fraction after further purification by two centrifugations at $150 \times g$ for 90 s. The cells were suspended at 5×10^6 cells/ml in Medium A. Hepatocytes and NPLCs with more than 90% viability, assessed by trypan blue exclusion, were used for the experiments. In our previous study (Yagi et al., 1995) cells were further purified by Percoll density gradient to determine the cell type responsible for the stimulating effect, the step was omitted not to decrease the yield of NPLCs in this study.

Bone marrow was isolated from rat femurs by flushing out with Hank's solution using a 22-gauge needle and dispersed by passing it through the needle. Bone marrow cells were centrifuged at 1200 × *g* for 5 min and the resulting pellet was suspended with 0.2% NaCl. After pipetting for 20 s and adding an equal amount of 1.6% NaCl, cells were centrifuged at 1200 × *g* for 5 min. The bone marrow cells were washed once and resuspended with RPMI 1640 supplemented with 10% FBS.

To prepare peritoneal exudate macrophages, rats were injected i.p. with 10% of their weight of 10% casein solution and sacrificed on the third or fourth day. After injecting i.p. 10% of their weight of Hank's solution containing 10U/ml heparin, peritoneal cells were collected and centrifuged at 1200 × *g* for 5 min. The resulting pellet was suspended with 0.2% NaCl. After pipetting for 20 s and adding an equal amount of 1.6% NaCl, cells were centrifuged at 1200 × *g* for 5 min. The peritoneal exudate macrophages were washed once and resuspended with RPMI1640 supplemented with 10% FBS.

Culture and differentiation of bone marrow cells

L-929 cells were cultivated with RPMl1640 supplemented with 2.5% FBS for 7 d. WEHI-3 was cultivated with RPMl1640 supplemented with 1.5% FBS for 5 d. The culture supernatants were filtrated through 0.22 μm membrane filters. The L-929 and WEHI-3 conditioned media were stored at –20 °C and used as the sources of crude M-CSF and lL-3, respectively. Bone marrow cells were cultivated with RPMI1640 supplemented with 10% FBS in the presence of M-CSF and IL-3 solutions, each at a concentration of 10%.

Hierarchical co-culture

A mixture of 0.3% collagen (Type l-A, Nitta Gelatin, Yao, Osaka, Japan) solution and 10 × concentrated medium at a ratio of 8:1 (v:v) was stirred gently on ice. Reconstitution buffer in which 0.477 g of HEPES was dissolved in 10 ml of 0.08N NaOH was added into the mixture to adjust the pH to 7.4. The ratio of the mixture to the buffer was 9:1 (v:v). Cells for the lower layer were seeded into a 12-well polystyrene culture dish (Sumilon, Sumitomo Bakelite, Tokyo, Japan). After cultivation for 2 h at 37 °C, 350 μl of the prepared collagen solution was added per well, on top of the cell layer and incubated for 1 h. Cells for the upper layer were then seeded onto the collagen.

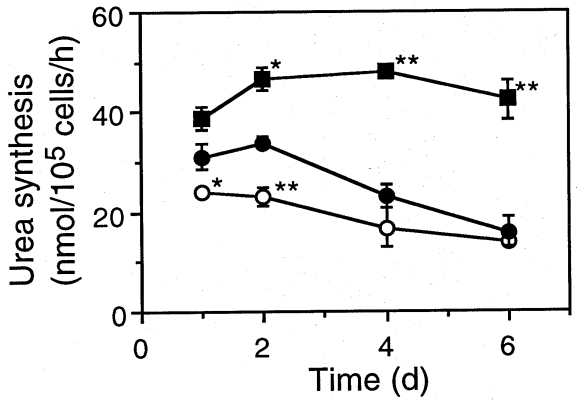

Figure 1. Stimulation of urea synthesis by hepatocytes in co-cultures with NPLCs. Hepatocytes and NPLCs were seeded at densities of 5 × 10^4 and 5 × 10^5 cells, respectively, in a monoculture of hepatocytes (○), a monolayer co-culture (●), and a hierarchical co-culture (■). Cells were incubated with WE containing 10^{-9}M insulin, 10^{-9}M dexamethasone, 5 ng/ml EGH, and 10% FBS. At the times indicated, cells were incubated for 4 h with Medium A supplemented with 2 mM NH_4Cl. Values are the means ± S.D. of three experiments. Asterisks indicate significant differences from the monolayer co-culture (*, p<0.01, **, p<0.001).

Esterase staining

Specimens were fixed for 30s with a solution containing 200 mg/l Na_2HPO_4, 1g/l KH_2PO_4, 45% acetone, and 25% formalin, rinsed with distilled water, and air dried. They were then incubated at room temperature for 45 min in a staining solution containing 1 g/l α-naphthyl butyrate, 2 g/l pararosaniline, and 2 g/l $NaNO_2$, and finally rinsed with water, and air dried.

Asssay

Induction of TAT was performed according to the method of Nakamura et al. (Nakamura et al., 1981). Cells were incubated in appropriate medium containing 10 μM dexamethasone and 0.1 mM dibutyryl cyclic AMP for 6 h, and the TAT activity was then assayed essentially as described by Granner and Tomkins (Granner et al., 1970). The amount of urea was measured by the method of Ormsby, (Ormsby, 1942) after incubation with 2 mM NH_4Cl for 4 h.

Results

Rat hepatocytes were cultured in polystyrene dishes under three conditions – alone in a monolayer, and in co-cultures with NPLCs both in a monolayer and

in a hierarchical configuration. Maintenance of urea synthesis in the hepatocytes was examined under each culture condition. As shown in Figure 1, the hierarchical co-culture, in which hepatocytes and NPLCs were separated by a collagen layer, maintained the highest activity among the three conditions. The activity of hepatocytes in the monolayer co-culture was maintained for 2d, after which it gradually decreased to approach that of hepatocytes cultured alone. Detached hepatocytes were observed at 3 d in the monolayer co-culture, probably due to the action or growth of NPLCs. Hepatocytes and NPLCs were located randomly and any cluster of the hepatocytes was not observed in the monolayer co-culture. The activity maintenance effect of NPLCs and a hierarchical co-culture were next examined with the rat hepatoma cell line H4-II-E-C3. The induction of TAT, one of the liver-specific functions, was used as an index because H4-II-E-C3 cells retained little urea synthesis activity. First various numbers of NPLCs and H4-II-E-C3 cells were co-cultured for 24 h in a monolayer and TAT was then induced for 6 h in the presence of dexamethasone and dibutyryl cAMP. As shown in Figure 2, the effect of the NPLCs was dose-dependent. The soluble factor (or factors) secreted from the NPLCs or cell-to-cell contact was thus shown to be effective with the hepatoma cell line as well as normal hepatocytes. In a hierarchical co-culture, NPLCs further stimulated TAT induction in H4-II-E-C3 cells (Figure 3). Since very little TAT was induced in NPLCs, hepatocyte contamination in the fraction appears to have been negligible. These results indicated that it was effective toward cells with extensive proliferation ability *in vitro*. For both hepatoma cells and hepatocytes, the hierarchical configuration was more effective than monolayer cultures.

In our previous work, we found that Kupffer cells, which are resident macrophages in the liver, were responsible for the stimulation of TAT induction in hepatocytes (Yagi et al., 1995). The stimulatory effect of macrophages from peritoneal exudate on TAT induction in hepatocytes was thus examined (Figure 4). No TAT was found to be induced in the macrophages themselves. Dose-dependent stimulation was observed in a monolayer co-culture of hepatocytes and macrophages isolated from peritoneal exudate (data not shown). Significantly stronger stimulation than that in the monolayer co-culture was observed in a hierarchical co-culture (see Figure 4). These findings suggested that macrophages differentiated *in vitro* from bone marrow cells or a macrophage-like cell line could be used as cell sources for hierarchical co-cultures.

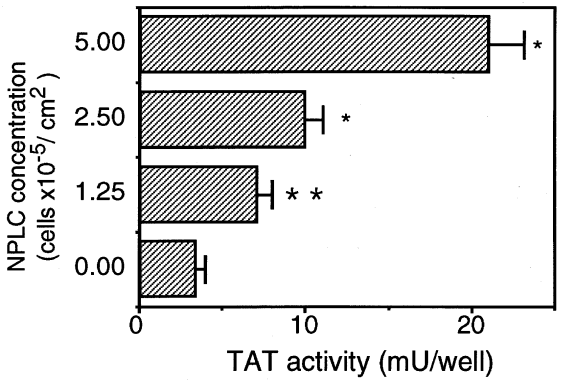

Figure 2. Stimulation of TAT induction in H4-II-EC3 cells by NPLCs in monolayer co-culture. H4-II-EC3 cells were seeded at a density of 1.67×10^4 cells/ml and co-cultured with the indicated densities of NPLCs for 48 h. TAT was then induced as described in Materials and methods. Values are the means ± S.D. of three experiments. Asterisks indicate significant differences from the monolayer co-culture (*, $p<0.001$, **, $p<0.005$).

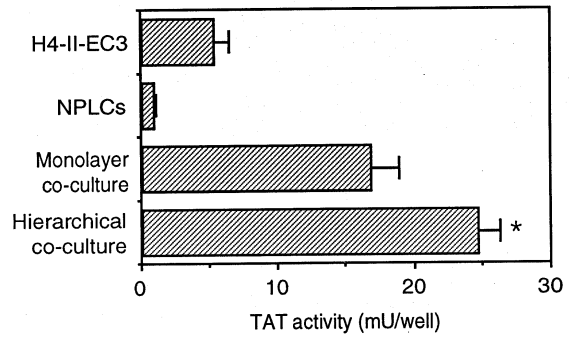

Figure 3. Effectiveness of hierarchical configuration in co-culture of H4-II-EC3 cells and NPLCs. H4-II-EC3 cells and NPLCS were seeded at densities of 1.67×10^4 and 5×10^5 cells, respectively, in each culture. TAT was induced after 48 h of cultivation. Values are the means ± S.D. of three experiments. The asterisk indicates a significant difference from the monolayer co-culture (*, $p<0.01$).

When the various concentration of macrophage-like cell line J774A.1 were co-cultured with hepatocytes in a monolayer, hepatocytes detached within 2 days. Although hepatocytes were co-cultured with J774A.1 cells in a hierarchical configuration and induced TAT, the activity was same as that in hepatocyte monoculture (data not shown). The immortalized macrophage appears to lose the ability of secreting the effective factor. We then investigated the use of bone marrow-derived macrophages. Bone marrow cells were isolated and incubated with IL-3 and M-CSF to obtain macrophages *in vitro*, as described in Materials and methods. After incubation for 5d, the

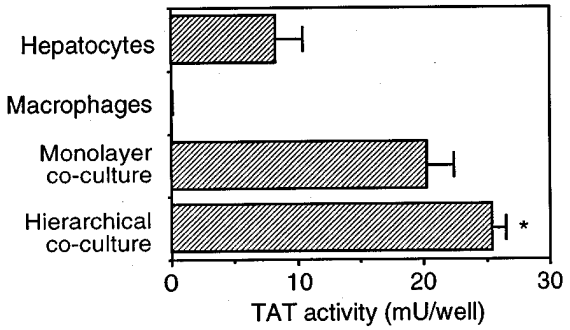

Figure 4. Stimulation of TAT induction in hepatocytes by macrophages from peritoneal exudate. Hepatocytes and macrophages were seeded at densities of 5×10^4 and 5×10^5 cells, respectively. TAT was induced after 48 h of cultivation. Values are the means ± S.D. of three experiments. The asterisk indicates a significant difference from the monolayer co-culture (*, $p<0.05$).

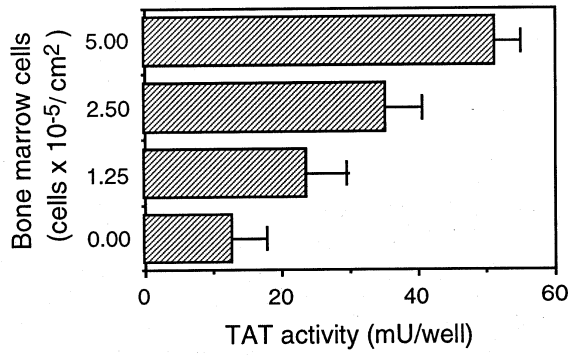

Figure 5. Stimulation of TAT induction in hepatocytes by bone marrow cells in monolayer co-culture. Hepatocytes were seeded at a density of 5×10^4 cells/ml and co-cultured with the indicated densities of bone marrow cells. TAT was induced after 48 h of cultivation. Values are the means ± S.D. of three experiments.

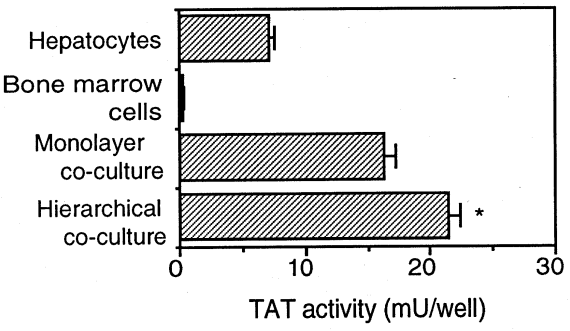

Figure 6. Effectiveness of hierarchical configuration in co-culture of hepatocytes and bone marrow cells. Hepatocytes and bone marrow cells were seeded at densities of 5×10^4 and 5×10^5 cells, respectively, in each culture. TAT was induced after 48 h of cultivation. Values are the means ± S.D. of three experiments. The asterisk indicates a significant difference from the monolayer co-culture (*, $p<0.01$).

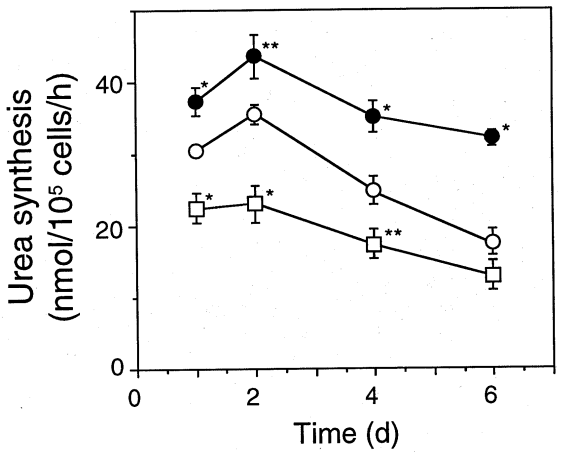

Figure 7. Stimulation of urea synthesis by hepatocytes in co-cultures with bone marrow cells. Hepatocytes and bone marrow cells were seeded at densities of 5×10^4 and 5×10^5 cells, respectively, in a monoculture of hepatocytes (□), a monolayer co-culture (○), and hierarchical co-culture (●). Cells were incubated with the medium containing 10^{-9}M insulin, 10^{-9}M dexamethasone, 5 ng/ml EGF, and 10% FBS. At the times indicated, cells were incubated for 4 h with Medium A supplemented with 2 mM NH_4Cl. Values are the means ± S.D. of three experiments. Asterisks indicate significant differences from the monolayer co-culture (*, $p<0.01$, **, $p<0.02$).

number of cells increased and the appearance of differentiated macrophages was confirmed by esterase staining (data not shown). Hepatocytes were co-cultured in a monolayer with various numbers of bone marrow cells containing differentiated macrophages. The TAT induction was stimulated in a dose-dependent manner as shown in Figure 5. When hepatocytes and the macrophages were co-cultured hierarchically, further stimulation of TAT induction was observed (Figure 6). No TAT was induced in the bone marrow cells. The effect of macrophages differentiated *in vitro* from bone marrow cells on urea synthesis in hepatocytes was then examined to ascertain whether their action is specific to TAT induction. Compared with a hepatocyte monoculture, urea synthesis activity in hepatocytes increased in monolayer and hierarchical co-cultures with mactophages derived from bone marrow. The results are shown in Figure 7. Throughout the 6-d incubation, the activity in the hierarchical co-culture was the most stably maintained among three conditions tested (see Figure 7). It was therefore demonstrated that bone marrow cells could be used as a source for the stimulation and maintenance of liver functions in hepatocytes. A combination of the hepatoma cell line and bone marrow cells, both of which have proliferation ability *in vitro*, was then examined. Figure 8 shows that in both

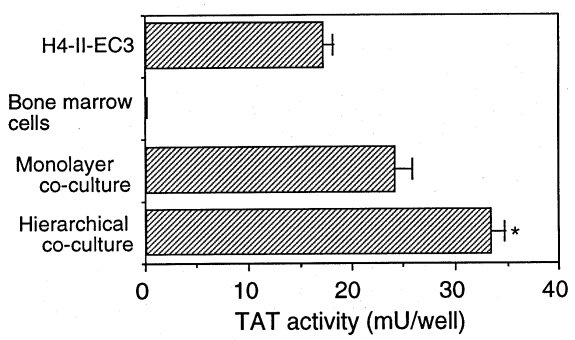

Figure 8. Stimulation of TAT induction in co-culture of H4-II-EC3 and bone marrow cells. H4-II-EC3 and bone marrow cells were seeded at densities of 1.67×10^4 and 5×10^5 cells, respectively. TAT was induced after 48 h of cultivation. Values are the means $\pm$ S.D. of three experiments. The asterisk indicates a significant difference from the monolayer co-culture (*, $p<0.01$).

monolayer and hierarchical co-cultures, bone marrow cells did have a stimulatory effect on TAT induction in H4-II-E-C3 cells.

Discussion

Hierarchical co-culture had a stimulatory effect superior to that of monolayer co-culture on TAT induction and urea synthesis in hepatocytes. In monolayer co-culture, the soluble factor(s) secreted from NPLCs easily diffused into the medium and therefore had less chance to make contact with hepatocytes. We previously found that when NPLCs were seeded in the upper layer of a hierarchical configuration instead of in the bottom layer, the stimulatory effect decreased to almost the same level as that of monolayer co-culture (Yagi et al., 1995) because the effective soluble factor(s) from the NPLCs dispersed into the medium as well as into the collagen layer. The stimulation level of a hierarchical co-culture also decreased when a transwell culture dish (Corning Coster, NY, U.S.A.), the bottom of which is made of membrane filter, was used (data not shown). It appears that in this case soluble factor(s) from the NPLCs in the bottom layer penetrate the filter into the medium, and the amount reaching the hepatocytes was insufficient. Thus, a hierarchical configuration in which hepatocytes and NPLCs are respectively seeded in the upper and lower layers is seen to be the most efficient for mutual communication through soluble factor(s). The function of the H4-II-E-C3 cell line, which is derived from Reuber H-35 hepatoma (Tsukada et al., 1979) and retains many liver functions (Tsukada, et al., 1985), was also stimulated by the co-culture with NPLCs. This result is considered to be significant, because an immortalized cell line might be a potential cell source for a BAL system. When co-cultured in a monolayer with the macrophage cell line J774A.1, hepatocytes were detached from the dish by the overwhelming growth of J774A.1 during 48-h cultivation, whereas they were stably cultured and maintained their function in a hierarchical co-culture. Separation by a collagen layer thus appears to protect hepatocytes from the unfavorable effects of rapidly growing fibroblasts present in NPLCs.

Although NPLCs comprise only 30% of the cell population in the liver, a larger number of NPLCs than hepatocytes was found to be necessary for stimulation. This poses the problem of how to collect sufficient cells for the production of an effective liver support device. Macrophages obtained from peritoneal exudate could stimulate the function of hepatocytes, although some heterogeneity exists between Kupffer cells and peritoneal macrophages (Laskin et al., 1988). Therefore, other sources of macrophages besides Kupffer cells were tried as possible candidates for co-culture with hepatocytes. The macrophage-like cell line J774A.1, which rapidly proliferates *in vitro* and produces interferon (Salo et al., 1985) and prostaglandin D_2 (McGuire et al., 1985), could be co-cultured hierarchically, but no stimulatory effect on hepatocytes was observed. However, bone marrows cells treated with IL-3 and M-CSF were shown to stimulate and maintain hepatocyte functions. Also conditioned medium prepared from treated bone marrow cells stimulated urea synthesis activity in hepatocytes (data not shown). This finding, together with the results of the hierarchical co-cultures, clearly shows a soluble factor(s) to be responsible, for the stimulation, and cell-to-cell contact to be not important. But there might have some possibility that cell-to-cell contact between hepatocytes on collagen gel contribute to increase the susceptibility to the soluble factor through dynamic signal transduction expressed by three dimensional organization. Only macrophage colonies were reported to develop, when bone marrow cells were cultured with M-CSF (Metcalf, 1989). However, the cell species secreting the effective soluble factor(s) is not clear, because IL-3 is a multilineage hematopoietic growth factor which can promote the proliferation and development of progenitor cells in granulocyte-macrophage, erythroid, eosinophil, megacaryocytic, mast cell, and basophilic lineages (Bazill, et al., 1983). Ihle, et al., 1983). Besides macrophages, basophils and

eosinophils might be contained in treated bone marrow cells. Identification of the effective factor and the cells producing it is necessary to elucidate the stimulation mechanism. When freshly isolated bone marrow cells were co-cultured with hepatocytes, stimulation of TAT induction was observed, although to a lesser extent than in the case of treated cells (data not shown). During co-culture, bone marrow cells might differentiate into macrophages and secrete an effective soluble factor(s), because hepatocytes have been reported to secrete M-CSF (Tsukui et al., 1992)

A BAL system for hierarchical co-culture has been constructed in the form of a multi-plate module (Nakajima et al., 1991), employing very thin glass plates set up in parallel, showing the feasibility of using the hierarchical configuration in a practical device. Since it is necessary to obtain a large number of functioning hepatocytes, approximately 200 g per patient, in order to use such a BAL system for widespread therapy, either a human cell line or the liver of animals such as pigs appear to be promising sources of hepatocytes, although the immune response to secreted compounds has to be considered before the clinical trial. When the hepatoma cell line is used for BAL, special attention should be made to avoid the migration into patient's body. The plasma from the patient should contact with hepatoma cell line through semi-permeable membrane. This study has demonstrated that the co-culture of bone marrow cells and a hepatoma cell line, both of which have proliferation ability *in vitro*, can be effective in stimulating liver functions. As cells derived from porcine or human must be used for BAL system, the experiments to confirm the effectiveness of the co-culture are currently under way using porcine cells.

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