

A liver-derived immunosuppressive factor is an arginase: identification and mechanism of immunosuppression

Yasuo OHTANI¹, Mineyoshi HIYOSHI^{2, 6}, Tomoichi OHKUBO^{2, 3}, Kimiyoshi TSUJI², Masao HAGIHARA², Hisao NAKASAKI¹, Hiroyasu MAKUUCHI¹, Naruhiko NAGATA⁴, Tetsuya MINE⁴, Shigeo TAKADA², Masaichi YAMAMURA² and Michio TSUDA^{2, 5}

¹ Department of Surgical Medicine, ² Department of Molecular Life Science, ³ Division of Teaching and Research Supporting Center, ⁴ Department of Internal Medicine, and ⁵ Department of Medical Education, Tokai University School of Medicine, Isehara, Kanagawa, 259-1193; and ⁶ Education and Research Projects for Cutting-Edge Basic Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, 770-8503, Japan

(Received 6 November 2006; and accepted 24 November 2006)

ABSTRACT

We found a substance in culture medium of neonatal pig liver fragments, which suppresses an immune response monitored by ³H-thymidine incorporation using phytohemagglutinin (PHA)-stimulated lymphocytes. We named it as an immunosuppressive factor (ISF). To purify ISF, ammonium sulfate fractionation, DE52, SP-Sephadex, hydroxyapatite, blue Sepharose, heparin Sepharose and Superdex gel filtration columns were used. Using these purification procedures, ISF was purified 1,254-fold, with 9.2% recovery, from the culture medium of neonatal pig liver fragments, and was identified as arginase by its biochemical characteristics including molecular size, amino acid sequences of digested peptides and expression of arginase activity. The addition of ISF caused to decrease in arginine concentration in culture medium and at the same time DNA synthesis was suppressed dose-dependently, both of which were recovered by the addition of NOHA (N^G-hydroxy-L-arginine), an arginase inhibitor. In addition, the depletion of arginine in culture medium also led to the inhibition of DNA synthesis. These results led us to the conclusion that immunosuppressive effect of ISF was due to arginase activity that decreased arginine concentration in culture medium, not to another function of ISF.

Hepatocellular transplantation is considered to be a useful therapy for acute and chronic liver failure induced by viral infection, drugs or surgical resection of a large part of the liver (7, 11, 18). We have previously reported the effect of transplanting small fragments of fetal liver into the omentum in rats with D-galactosamine-induced liver failure and in 90 % hepatectomized rats (8, 14, 16, 19). When fetal pig liver fragments were grafted in these rats

with acute liver failure, the grafted liver survived for 14 days judging from histological examinations and the rate of survival of these rats was higher than that of control non-grafted groups. The same results were confirmed when the grafts were neonatal liver fragments. From the fact that fetal or neonatal liver are immature in function and that liver fragments grafted were a few, we considered that the effect may be not only due to compensation of

Address correspondence to: Dr. Michio Tsuda, Department of Medical Education, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193 Japan
Tel: +81-463-93-1121 (ext.2552), Fax: +81-463-96-4828
E-mail: mtsuda@is.icc.u-tokai.ac.jp

Abbreviation: ISF; immunosuppressive factor, HPF; hepatocyte proliferation factor, DMEM; Dulbecco's modified Eagle's medium, PBS; phosphate-buffered saline, SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis

recipient liver function, but to some secreted factors which enhance liver regeneration and liver function in the recipient, or has an immunosuppressive effect for keeping the grafted liver fragment intact longer.

When neonatal liver fragments were cultured, we found two kinds of factors in the culture medium. One stimulates DNA synthesis of rat primary cultured hepatocytes, and the other suppressed an immune response monitored by ^3H -thymidine incorporation using PHA-stimulated lymphocytes. The former was named hepatocyte proliferation factors (HPFs), and the later was immunosuppressive factor (ISF). We have already reported the chemical and biological characterization of HPFs, which were capable of stimulating DNA synthesis in primary hepatocytes and supposed to be novel factors different from hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor- α (TGF- α) and heparin-binding EGF-like growth factor (HB-EGF) (9).

In this paper we report complete purification and some biological characterization of ISF.

MATERIALS AND METHODS

Materials. The following compounds were purchased: RPMI 1640, DMEM, Dulbecco's PBS(-) (Nissui, Tokyo, Japan), EX-CELL 610-HSF medium and fetal bovine serum (FBS)(JRH Biosciences, Lenexa, USA), Liver Perfusion Medium, Liver Digest Medium, Hepatocyte Wash Medium, trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA), penicillin-streptomycin, gentamicin (GIBCO, New York, USA), NOHA (N^G -hydroxy-L-arginine), bovine insulin, porcine glucagon, dexamethasone (Sigma, St. Louis, USA), DE52 (Whatman Inc, Clifton, USA), hydroxyapatite (Bio Rad, Hercules, USA), blue Sepharose CL-6B, heparin Sepharose CL-6B, HiTrap Q column, Superdex 200 column, methyl- ^3H -thymidine (Amersham Pharmacia Biotech, Piscataway, USA), Yorkshire pigs (Cimco Co., Tokyo, Japan), cultured cells including HepG2 cells (human hepatocellular carcinoma), JTC-12 cells (monkey kidney), BALB/3T3 cells (embryonic mouse fibroblast) and IEC-6 cells (rat small intestinal epithelial cells) (Riken, Wako, Japan). Animal experiments were performed in accordance with guidelines of Tokai University and NIH for the care and use of laboratory animals.

Culture of neonatal pig liver fragments. Neonatal (1–2 day after birth) pigs were anesthetized by injecting a mixture of atropine sulfate, ketalar (ketamine hydrochloride) and stresnil (azaperone) to

muscular tissue and diethyl ether was used as an additional gaseous anaesthetic. The liver was perfused with 100 mL DMEM (pH 7.4, 37°C) supplemented with 20 mM HEPES, penicillin 100 U/mL, streptomycin 100 $\mu\text{g}/\text{mL}$ and gentamicin 50 $\mu\text{g}/\text{mL}$ by cannulation from the umbilical vein (flow rate 15 mL/min). The liver was removed and cut into 2–3 mm squares with a disposable scalpel in a glass dish on ice. About 5 mL of these liver fragments were cultured in 30 mL of EX-CELL 610-HSF serum free medium supplemented with 20 mM HEPES, pH 7.4, at 37°C. After 16 h, the culture medium was collected and used as the starting material for purification.

Separation of human leukocytes. Human peripheral blood with 20 units/mL heparin was collected, mixed with an equal volume of plasmagel (6) and left standing for 20–30 min at 37°C. Leukocytes in the upper layer were carefully collected and centrifuged. The leukocytes were washed with RPMI 1640 and used for the measurement of an immunosuppressive activity.

Measurement of immunosuppressive activity. The lymphocytes ($4 \times 10^4/\text{mL}$) in RPMI 1640 medium containing 10% FBS and 20 mM HEPES-buffer, pH 7.4 were placed in a well of microplate and incubated for 16 h at 37°C. Then PHA was added to the plate with or without ISF fraction, and the microplate was further incubated for 72 h. Four hours before harvesting the cells, ^3H -thymidine, 3.4×10^4 Bq was added. The cells were harvested onto glass fiber filter. The filter was extensively washed with PBS and dried. The incorporation of ^3H -thymidine into lymphocytes was measured by a liquid scintillation counter. One unit of ISF activity was defined as the ability that causes 90% inhibition of ^3H -thymidine incorporation.

Purification Procedure. ISF activity was observed in the condition medium obtained from both neonatal and fetal liver fragments. As the conditioned medium from neonatal pig liver fragments had about the same activity with that from fetal pig liver fragments, and neonatal pig liver is bigger and can be obtained more easily than fetal pig liver, we used neonatal material for purification.

To one liter of the condition medium obtained from 5 neonatal pigs, solid ammonium sulfate was added until 85% saturation was reached. The solution was centrifuged at $13,500 \times g$ for 35 min and the precipitate was dissolved in 20 mM Tris-HCl

buffer, pH 8.0 (the basal buffer). The solution was extensively dialyzed against basal buffer and applied to a DE52 column (5.6×10 cm) previously equilibrated with basal buffer. The column was washed with the same buffer. To non-adsorbed active fractions, solid ammonium sulfate was added until 85 % saturation was reached. The precipitate was dissolved in 20 mM Tris-HCl buffer, pH 8.0 (basal buffer). The solution was extensively dialyzed against basal buffer and applied to a SP-Sephadex column (2.6×5 cm) previously equilibrated with basal buffer. Non-adsorbed fractions were applied to a hydroxyapatite column (2.6×5 cm) previously equilibrated with basal buffer. The column was washed with 80 mM potassium phosphate buffer, pH 7.15, and then eluted with 200 mM potassium phosphate buffer. The active fractions were pooled, concentrated with Vivaspin concentrator (Vivascience, UK), and dialyzed against basal buffer.

This solution was applied to a blue Sepharose CL-6B column (1.2×5 cm) previously equilibrated with the basal buffer washed. The non-adsorbed fractions were concentrated with Vivaspin concentrator and applied to heparine Sepharose column (1.2×3 cm). The non-adsorbed fraction was concentrated and applied to a Superdex 200 column (1.0×60 cm) equilibrated with basal buffer containing 0.15 M NaCl. ISF activity was eluted as a sharp single peak (Fig. 1). The molecular weight was estimated at 140–160 kDa. This final preparation was used to examine the chemical and biological characteristics of ISF.

SDS-PAGE analysis, amino acid sequence and measurement of arginase activity. SDS-PAGE was performed as described by Laemmli (10). A main protein band in SDS gel was extracted by 50 mM Tris-HCl buffer, pH 9.0 and digested by Lys C (Wako Pure Chemicals Industries, Ltd, Osaka, Japan). The digestive solution was applied to a column of

SMART system for peptide mapping (Amersham Pharmacia Biotech, Piscataway, USA). Peptides from the column were used in sequence analysis by the standard method using Shimadzu PSSQ21 Protein Sequencer.

Arginase activity was measured as the amount of urea from arginine according to the method of Coulombe and Favreau (5).

RESULTS

Purification of ISF

To purify ISF, ammonium sulfate fractionation, DE52, SP-Sephadex, hydroxyapatite, blue Sepharose, heparin Sepharose and Superdex 200 gel-filtration columns were used. Using these purification procedures, ISF was purified 1,254-fold, with 9.2% recovery, from the condition serum-free culture medium of neonatal pig liver fragments (Table 1).

SDS-PAGE analysis, amino acid sequence and measurement of arginase activity

The molecular size of ISF was estimated as 140–160 kDa by Superdex 200 gel-filtration. SDS-PAGE showed a single band with a molecular size of 37 kDa (Fig. 1), which indicated ISF is a tetramer with 4 identical subunits.

A main single protein band in SDS gel was extracted by 20 mM Tris-HCl buffer, pH 9.0, digested by Lys C and then applied to SMART system. Four out of peptides separated were used in amino acid sequence analysis. These results showed that amino acid sequence of every 4 peptides analyzed is identical or very similar to that of human arginase as shown in Fig. 2.

Inhibition of ^3H -thymidine incorporation by ISF

The incorporation of ^3H -thymidine by PHA-stimulated lymphocytes was dose-dependently suppressed by the addition of ISF. The addition of 0.5 and 2

Table 1. Purification of ISF

Purification Steps	Total activity (units)	Recovery (%)	Specific activity (unit/mg)	purification (fold)
Culture medium*	103000	100	11.4	1
DE52	90000	87	153.8	13.5
SP-Sephadex	25000	24.3	416.7	36.6
Hydroxyapatite	16000	15.5	1600	140
Blue-Sepharose	10200	9.6	6670	585
Heparin-Sepharose	12080	11.7	13130	1152
Superdex 200 Gel-filtration	9500	9.2	14290	1254

*Culture medium (about 1 L) from 5 new-born pig livers.

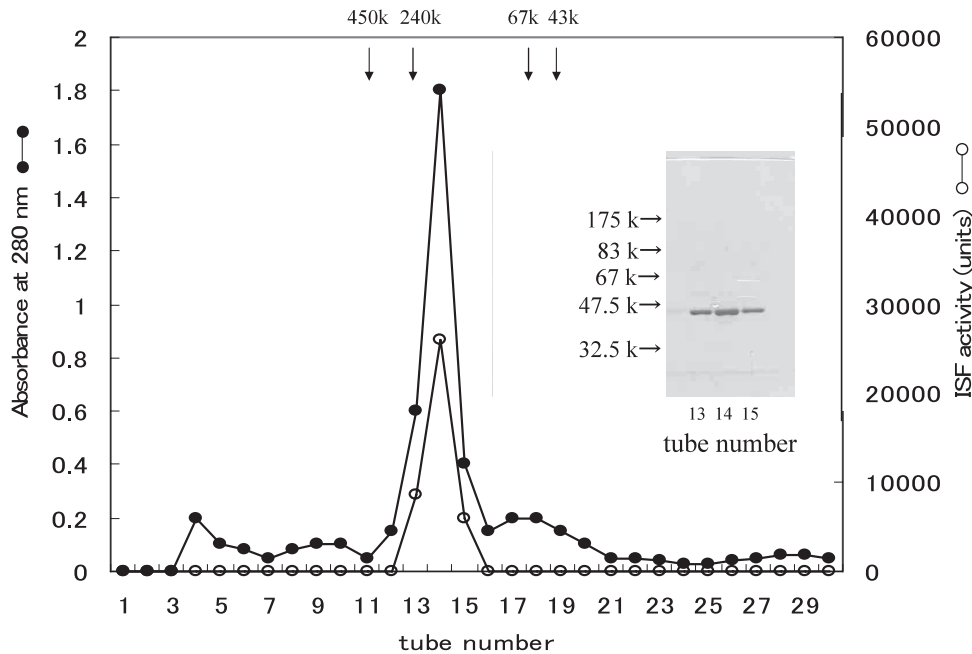


Fig. 1 Superdex 200 gel-filtration pattern and SDS-PAGE analysis. The sample solutions of tube number 13, 14 and 15 from Superdex 200 gel-filtration column were applied to SDS-PAGE analysis.

unit of ISF caused 50%, 90% suppression, respectively (Fig. 3). The arginine concentration in culture medium decreased from 1,050 μM to less than 5 μM 72 h after the addition of 0.5 unit ISF (Fig. 3). The addition of 0.5 mM NOHA, an arginase inhibitor, completely recovered the suppression by ISF (from 0.5 to 2 units) and, at the same time, the decrease of arginine concentration was partially inhibited (Fig. 3).

Arginine dependency for ^3H -thymidine incorporation in various cells

Arginine dependency studies using PHA-stimulated lymphocytes were shown in Fig. 4a, indicating that the lymphocytes needed at least 23 μM arginine for full incorporation of ^3H -thymidine. This conclusion was coincident with the result of Fig. 3. In addition, arginine dependency of various cells for DNA synthesis was examined (Fig. 4b-e). Arginine concentrations for half-maximum DNA synthesis were in the range of 2.9–5.8 μM for HepG2, IEC-6, and JTC-12 cells, which are very similar to human lymphocytes. In BALB/3T3 cells, 2.9 μM arginine showed max cell growth, and the cells were grown even in the absence of arginine in culture medium as shown in Fig. 4e. The sensitivity to ISF of HepG2, IEC-6, and JTC-12 cells was also similar to that of human lymphocytes; the suppression by 1 unit ISF were 99.3,

99.4, 94.4%, respectively, but the suppression in BALB/3T3 cells was 57.5 % of the control in the presence of 1 unit of ISF. Primary hepatocytes were resistant to ISF, because the suppression was only 23% of the control, even when 2 units of ISF was added to culture medium (not shown).

DISCUSSION

Several liver-derived immunosuppressive factors from human beings (2, 4, 15), mouse (1), rat (12, 17) and dog (3) that inhibit lectin-induced lymphocyte proliferation and mixed lymphocyte reaction *in vitro* have been identified. Brusdeilins and Shinomiya *et al.* have characterized one of the immunosuppressive factors as arginase protein (2, 17). In the present study, we also found immunosuppressive activity in the condition culture medium obtained from neonatal liver fragments. This active factor was completely purified and identified as arginase by its biochemical characteristics including molecular size, amino acid sequences of digestive peptides and arginase activity, as described above.

Recently some proteins have been recognized as having additional functions besides their original activity. Therefore we investigated whether the immunosuppressive effect of ISF is due to arginase activity or to a new function of arginase. The addition

a) Amino acid Sequence of peptide fragments of ISF

	number of different amino-acids compared to human arginase
(K) DIVYIGLRD <u>VDPA</u> EHYI	1 /17
(K) YFSM <u>L</u> EVGK	2 /10
(K) VMEETFSYLLGRK	0 /14
(K) TGLLSGLDIMEVNPTLGK	0 /18

b) Amino acid Sequence of human arginase

```

MSSKPKPIEI IGAPFSKGQP RGGVEKGPAA LRKAGLVEKL KETEYNVRDH
GDLAFVDVFN DSPFQIVKNP RSVGKANEQL AAVVAETQKN GTISVVLGGD
HSMAGSISG HARVHPDLCV IWVDAHTDIN TPLTTSSGNL HGQPVAFLLK
ELKGKFPDVP GFSWVTPCIS AKDIVYIGLR DVDPGEHYII KTLGIKYFSM
TEVDKLGIGK VMEETFSYLL GRKKRPIHLS FDVDGLDPVF TPATGTPVVG
GLSYREGLYI TEEIYKTGLL SGLDIMEVNP TLGKTPPEVT RTVNTAVALT
LSCFGTKREG NHKPETDYLK PPK

```

Fig. 2 Amino acids sequence of ISF

Amino acids sequence of 4 peptides from digestive ISF were shown in (a). The sequence of human arginase was shown in (b). Underlines in (a) indicate different amino acid from that of human arginase. The parts of underlines in (b) are relative to identified amino acids in (a).

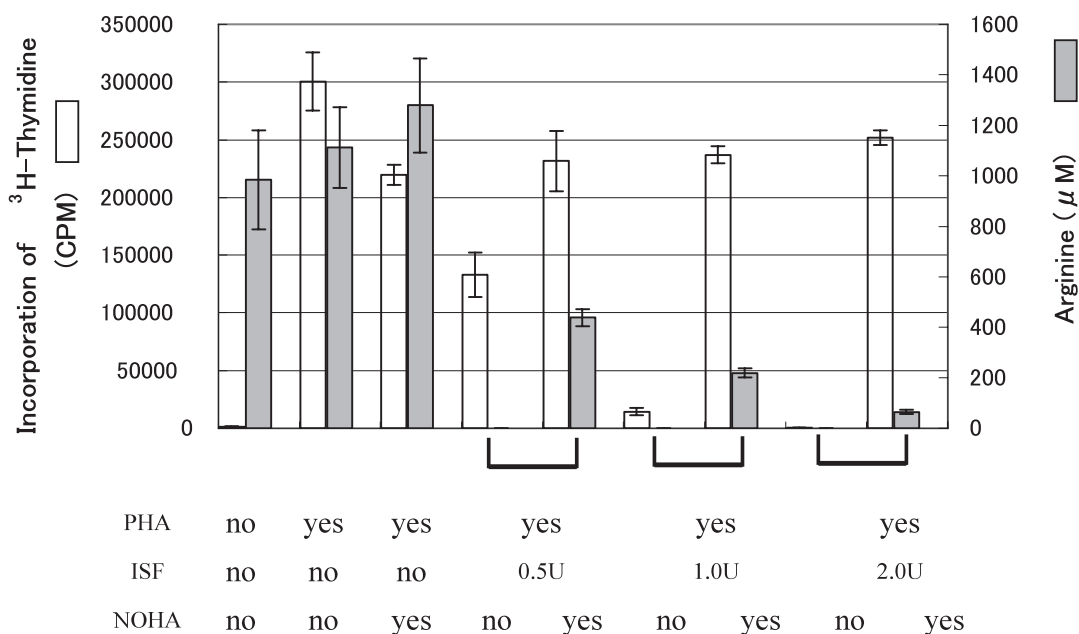


Fig. 3 The incorporation of ^3H -thymidine by PHA-stimulated lymphocytes and arginine concentration of culture medium. The lymphocytes (4×10^4 /mL) were incubated for 16 h at 37°C . Then PHA was added to the plate with or without ISF or/ and 0.5 mM NOHA, and the microplate was further incubated for 72 h. Four hours before harvesting the cells, ^3H -thymidine, 3.4×10^4 Bq was added. The incorporation of ^3H -thymidine into lymphocytes (closed bar) was measured by a liquid scintillation counter. The arginine concentration in the culture medium (open bar) was measured by amino acid analyzer.

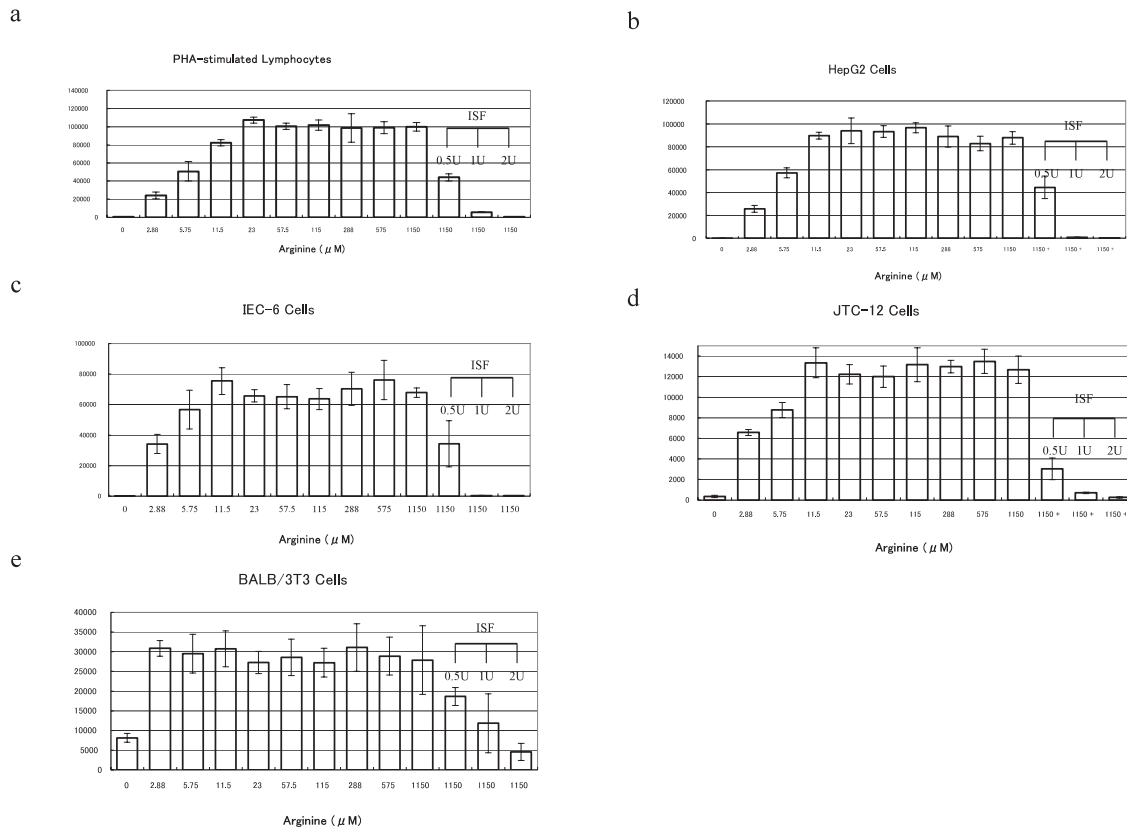


Fig. 4 Arginine dependency for ³H-thymidine incorporation in various cells

Arginine dependency of various cells, such as PHA-stimulated lymphocytes, HepG2 cells, IEC-6 cells, JTC-12 cells and BALB/3T3 cells, for DNA synthesis was examined. Cells were suspended in arginine-free medium contained various concentration of arginine with or without ISF. The cells were further incubated for 72 h. Four hours before harvesting the cells, ³H-thymidine, 3.4×10^4 Bq was added, then the incorporation of ³H-thymidine into cells was measured by a liquid scintillation counter.

of ISF caused a decrease in arginine concentration in culture medium and DNA synthesis of PHA-induced lymphocytes was suppressed dose-dependently, which were inhibited by the addition of NOHA, an arginase inhibitor. In addition, the limitation of arginine concentration in culture medium also led to the inhibition of DNA synthesis. These results support that the immunosuppressive effect of ISF was the result of depletion of arginine from culture medium, not to another function of ISF.

To examine whether this arginine requirement for DNA synthesis is specific for PHA-stimulated lymphocytes, we compared it between human lymphocytes and other cells including HepG2 cells, IEC-6 cells, JTC-12 cells, BALB/3T3 cells and primary rat hepatocytes. As shown in Fig. 4, the inhibition of DNA synthesis by ISF or arginine depletion is very sensitive to HepG2 cells, IEC-6 cells, JTC-12 cells. In primary hepatocytes, ISF had no effect to DNA synthesis and the depletion of arginine from culture

medium did not also cause any inhibition of their DNA synthesis (data not shown). Hepatocytes have arginine synthetic pathway including urea cycle, therefore exogenous arginine might not be needed. BALB/3T3 cells were also partially grown in the arginine-free medium, and showed the low sensitivity to ISF. These cells should also contain some arginine synthetic pathway. We conclude that arginine is essential for growth of not only lymphocytes but also cultured cell lines that do not have arginine synthetic pathways.

When fetal or neonatal pig liver fragments were grafted in rats with D-galactosamine-induced liver failure and in 90% hepatectomized rats, the rate of survival in these groups was higher than that in control non-grafted groups (8, 14, 16, 19). In addition, we found two kinds of biological factors from the culture medium of pig liver fragments, one is HPFs described before (9) and the other is ISF, arginase, reported in this paper. The activity of arginase is

relatively low in the rat fetus and rapidly increased after birth. Neonatal liver contained 30–50% arginase activity of rat and human adult liver (13). ISF, arginase, protects the immunological rejection of transplanting grafts and HPFs enhance the proliferation of remaining liver and transplanting liver fragments, leading to recover of liver function in the rats. Therefore, a part of the survival effect of liver fragments to rats with acute liver failure may be due to either or both factors, although the effect of arginine depletion by arginase, ISF, is not specific for lymphocytes. In addition, liver grafts in many species are rejected less aggressively than other organ graft. This observation may be explained as a result of immunosuppressive effect in the rejection reaction by circulating arginase from intact or damaged transplanted liver tissue.

Acknowledgments

We express our appreciation to Mr. Hideo Tsukamoto and Mr. Hiroshi Kamiguchi, Division of Teaching and Research Supporting Center, Tokai University, School of Medicine, for their technical suggestion. We are also grateful to Dr. Nadia El Borai for correcting the manuscript.

REFERENCES

1. Adler AJ, Kong AS and Friedman EA (1978) Effect of cell-free murine liver extract on lymphocyte blastogenesis in vitro. *Cell Immunol* **36**, 271–282.
2. Brusdeilin M, Hapke C, Huberts HH and Schumacher K (1983) Identification of the apparently lymphocyte-specific human liver-derived inhibitory protein (LIP) as cytoplasmic liver L-arginase. *J Immunol* **131**, 2427–2431.
3. Burlison RL, Ennulat MM, Gurman AH, Hubbel C and Barrett D (1979) Demonstration of a dog liver immunosuppressive factor active in vitro. *Transplantation* **28**, 318–322.
4. Chisari FV (1978) Regulation of human lymphocyte function by a soluble extract from normal human liver. *J Immunol* **121**, 1279–1286.
5. Coulombe JJ and Favreau L (1963) A new simple semimicro method for colorimetric determination of urea. *Clin Chem* **9**, 102–108.
6. Coulson AS and Chalmers DG (1964) Separation of viable lymphocytes from human blood. *Lancet* **18**, 468–469.
7. Habibullah CM, Syed IH, Qamar A and Taher-Uz Z (1994) Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. *Transplantation* **58**, 951–952.
8. Hagihara M, Shimura T, Takebe K, Munkhbat B and Tsuji K (1994) Iso and xeno fetal liver transplant therapy for the treatment of acute and chronic liver failure in rats. *Cell Transplantation* **3**, 29–30.
9. Hiyoshi M, Ohkubo T, Tsuji K, Hagihara M, Nakasaki H, Mukai M, Makuuchi H, Yamamura M and Tsuda M (2002) Hepatocyte proliferation factors from neonatal pig liver: Purification and characterization. *BioFactors* **16**, 1–14.
10. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
11. Makaowka L, Rotstein IE, Falk JA, Langer B, Nossal NA, Blendis LM and Philips M (1980) Reversal of toxic and anoxic induced hepatic failure by syngeneic, allogeneic and xenogeneic hepatocyte transplantation. *Surgery* **88**, 244–253.
12. Pizzo C, Lee D and Chisari FV (1982) Suppression of lymphocyte activation by a protein released from isolated perfused rat liver. *Hepatology* **2**, 295–303.
13. Raiha NC (1976) Developmental changes of urea-cycle enzymes in mammalian liver. In: *The Urea Cycle* (Grisolia S, Baguena R and Mayor F, eds.), pp261–272, Wiley Interscience, New York.
14. Sano N, Matsuno N, Sugimoto A, Seiki K and Tsuji K (1989) Fetal liver allo- and xenotransplantation in acute and chronic hepatic failure. *Transplant Proc* **21**, 2360–2361.
15. Schumacher K, Maerker-Alzer G and Wehmer U (1974) A lymphocyte-inhibiting factor isolated from normal human liver. *Nature* **251**, 655–656.
16. Shimura T, Hagihara M, Takebe K, Munkhbat B and Tsuji K (1995) Analysis of survival of allogeneic fetal liver fragments in rats. *Cell Transplantation* **4** (suppl), S45–S47.
17. Shinomiya T, Ohara T, Wada N, Omori A and Kamada N (1990) Rat liver arginase suppresses mixed lymphocyte reaction. *J Biochem* **107**, 435–439.
18. Sutherland DER, Numata M, Matus AJ, Simmonds RL and Najarian JS (1977) Hepatocellular transplantation in acute liver failure. *Surgery* **82**, 124–132.
19. Takebe K, Shimura T, Munkhbat B, Hagihara M, Nakanishi H and Tsuji K (1996) Xenogeneic (pig to rat) fetal liver fragment transplantation using macrocapsules for Immunoisolation. *Cell Transplantation* **5** (suppl), S31–S33.

