The *In Vivo* Effect of Hepatotrophic Factors Augmenter of Liver Regeneration, Hepatocyte Growth Factor, and Insulin-Like Growth Factor-II on Liver Natural Killer Cell Functions

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Abstract

Fine balanced sequential changes of the levels of circulating hepatotrophic factors are essential for normal liver regeneration. Our recent studies have indicated that liver-resident natural killer (NK) cells are important regulators of liver regeneration and have raised the possibility that hepatotrophic factors might mediate their activities through NK cells. In the present study, we assessed the effects of *in vivo* administration of three hepatotrophic factors (augmenter of liver regeneration [ALR], insulin-like growth factor-II [IGF-II], and hepatocyte growth factor [HGF]) on NK cells in normal rats. Each of the three, given over a 1-day period in doses known to produce hepatotrophic activity, induced inhibition of NK cell cytotoxic activities in the population of mononuclear leukocytes (MNL) in the liver, but not in MNL from the spleen or peripheral blood. In contrast to these results obtained by the whole animal treatment, the three molecules had no effect on NK cell functions when added to cultures of MNL from the livers, spleens, or blood of untreated rats. These data support and extend our previously advanced hypothesis that ALR and other hepatotrophic factors play an important role in liver regeneration by regional regulation of NK cells through some as-yet-unknown intermediary mechanism.

Hepatotrophic factors (Table 1) have been so characterized[1–15] by virtue of their ability to augment the hyperproliferative response induced by partial hepatectomy (PH) in rats[3,6] or dogs,[5] and to prevent the atrophy as well as augment the heightened cell renewal caused by portacaval shunt (Eck's fistula) in dogs.[2,4] Only two of the eight known hepatotrophic factors (hepatocyte growth factor [HGF] and transforming growth factor [TGF]-*α*) are primary mitogens capable of stimulating *de novo* hepatocyte proliferation *in vitro* without the requisite precondition of preexisting commitment to heightened cell renewal. Molecules with opposite effects have been called "antihepatotrophic."[16–18]

After PH, there is an acute temporary increase in the circulating levels of several of the endogenous hepatotrophic factors[1,2,4,5,19] and multiple gene expression in the liver.[20] We

have speculated that the hepatocyte stimulatory activity of augmenter of liver regeneration (ALR) and the other hepatotrophic factors may be mediated through or by nonparenchymal cells.[1,9,21] This possibility was reinforced by the observation during the acute phase of regeneration after PH in rats of temporary suppression of natural killer (NK) cell functions of liver mononuclear leukocytes (MNL), followed by their recovery at the termination of regeneration.[21] These changes occurred contemporaneously with mirror-image susceptibility of the regenerating hepatocytes to NK-induced lysis.

To test this immunologic hypothesis, we have determined in the present study whether the changes of NK cell function occurring after PH could be reproduced in unoperated naive rats by their treatment with three potent recombinant hepatotrophic factors chosen from the list in Table 1: IGF-II, HGF, and ALR. All three of these systemically administered growth factors were found to mimic all of the changes of spontaneous NK cell functions associated with PH, including the limitation of their effect to liver mononuclear leukocytes (MNL). Because these factors had no effect on cultures of liver MNL from naive rats, additional unknown extra- or intrahepatic mediator(s) may be involved in their hepatic growth regulation.

MATERIALS AND METHODS

Materials and Assays

Animals—Male Fischer F344 rats (180–200 g) were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA), and were housed in a specific pathogen-free animal facility of the Pittsburgh Cancer Institute for at least 20 days before the experiments and were fed with standard rat chow and water *ad libitum.*

Antibodies and Reagents—For phenotyping of rat MNLs, fluorescein isothiocyanate–labeled mouse monoclonal antibodies to rat T-lymphocyte surface antigens including anti-CD5 (OX19, immunoglobulin [Ig]G1) from Accurate Chemical and Scientific Corp. (Westbury, NY), and CD3 (G.4.1.A., IgG3) from Bioproducts for Science (Indianapolis, IN) were purchased. Monoclonal antibody against NKR-P1 (3.2.3, IgG1) was raised and conjugated with fluorescein isothiocyanate in our laboratory for the identification of NK cells. IgG isotope control antibodies were purchased from Becton Dickinson (San Jose, CA).

Rat recombinant (r)ALR expressed in transfected COS-1 cells[9] and rHGF were gifts from Toyobo Co., Ltd. (Ohtsu, Shiga, Japan), while rIGF-II was purchased from United States Biochemical (Cleveland, OH).

Isolation of Rat MNL—Liver, spleen, and blood MNL were isolated and purified, as previously described.[21] Briefly, liver-resident MNL were isolated, after enzymatic digestion of liver tissue, using metrizamide and Ficoll-Hypaque density gradient centrifugations. MNL from blood and spleen were separated using Ficoll-Hypaque density gradient centrifugations.

Immunofluorescent Staining and Flow Cytometry of MNL—The MNL were adjusted to a concentration of $0.25 \times 10^6/0.2$ mL in phosphate-buffered saline containing 0.1% (wt/vol) sodium azide and 1% fetal bovine serum (vol/vol). For direct single-color immunofluorescent staining, the cells were mixed with fluorescein isothiocyanate–labeled mAbs. Cells suspended in phosphate-buffered saline–azide buffer alone or in fluorescein isothiocyanate–conjugated IgG1 monoclonal antibodies (mAb) were used as negative controls. After incubation with the mAbs or reagents at 4°C for 30 minutes, the cells were washed two times with phosphate-buffered saline–azide buffer, resuspended in 1% (wt/vol) paraformaldehyde, and examined by single-color flow cytometry on a FACScan.[21] Data

were analyzed using the Reproman computer program (Flow Cytometry Analysis Software for Personal Computers, True Facts Software Inc., Seattle, WA).

Cytotoxicity Assays

A standard 4-hour ^{51}Cr-release microcytotoxicity assay was used to quantitate direct cytolytic activity against the NK-sensitive YAC-1 lymphoma cells and NK-resistant P815 mastocytoma cells, as previously described.[21]

Antibody-Dependent Cellular Cytotoxicity—After ^{51}Cr-labeling, P815 target cells were preincubated for 15 minutes at room temperature with rat anti-P815 antiserum (a gift of Dr. W.H. Chambers) at a subagglutinating concentration, washed twice, and mixed with the MNL.

Redirected Killing Via NKR-P1—MNL were preincubated with anti–natural killer receptor protein 1 (NKR-P1) mAb (0.1 μg/mL) at room temperature for 15 minutes. This procedure resulted in sensitization of NK cells in the population of MNL, as a consequence of the specific binding of this mAb to NKR-P1 receptor. The assay was continued by adding to the effector cells ^{51}Cr-labeled P815 target cells, which express cell surface Fc receptors and are able to ligate the Fc portion of anti–NKR-P1 mAb bound to NK cells, and thus to cross-link the NKR-P1 receptor, triggering cytolytic activity of NK cells.[22] P815 cells preincubated with rat serum obtained before immunization or with a nonreactive IgG_1 mAb served as negative control targets to antibody-dependent cellular cytotoxicity or redirected killing, respectively. Spontaneous release and maximum release were determined by incubating target cells without effectors in medium alone or in 5% Triton X-100, respectively. The spontaneous release was less than 10% for both YAC-1 and P815 target cells.

All of the foregoing assays were performed in triplicate. Radioactivity was counted in a gamma counter, and the percentage of specific lysis was determined according to the formula:

$$\% \text{ specific lysis} = \frac{[\text{mean cpm experimental release} - \text{mean cpm spontaneous release}]}{[\text{mean cpm maximal release} - \text{mean cpm spontaneous release}]} \times 100$$

Lytic units (LU) were calculated using a computer program developed by Pross et al.[23] One lytic unit was defined as the number of effector cells needed to lyse 20% of 5×10^3 target cells. LU per 10^7 effector cells were calculated on the basis of the following formula:

$$\frac{LU_{20}/10^7 \text{MNL}}{\% \text{NKR} - \text{P1}^{\text{bright}}\text{MNL}} \times 100$$

Experimental Design

***In Vivo* Effect of Hepatotrophic Factors on NK Cell Functions of Normal Nonoperated Rats—**Four doses of 125 ng rALR or rIGF-II were injected intramuscularly and four doses of 1,000 ng rHGF were given intravenously at 24, 18, 12, and 6 hours before the animals were killed. The animals were killed, and MNL were isolated from peripheral blood, spleen, and liver. The phenotype and cytotoxic activities of MNL were determined.

***In Vitro* Effects of Growth Factors on NK Cells in MNL Populations—**
Suspensions of MNL isolated from the blood, spleen, and liver of normal rats were prepared

in culture medium (RPMI 1640 plus 10% fetal bovine serum), which was supplemented with hepatotrophic factors rALR, rIGF-II, and rHGF singly or in combination. The cells were either assessed immediately or after 24 hours of culture at 37°C. Their cytotoxic activity was tested in the assays described above.

Statistical Analysis

Statistical significance of the results was calculated using Wilcoxon's signed rank pair and Mann-Whitney U tests. Differences were considered significant at $P < .05$.

RESULTS

Systemic Administration of Hepatotrophic Factors

Composition of MNL in the Liver and Peripheral Lymphoid Tissues—The percentages of NKR-P1^{bright} (NK) cells and CD5^{+} or CD3^{+} (T) lymphocytes in the liver, spleen, or blood of rats after administration of any of the hepatotrophic factors were not different from the saline controls (Table 2). The failure of treatment to influence changes of the proportions of MNL subpopulations, either in the liver or in the peripheral lymphoid tissues was similar to that after PH.[21]

NK Cell Functions—In contrast to the unchanged phenotype, the rALR, rHGF, and rIGF-II had significant and multiple suppressive effects on various NK cell functions (Table 3). The 24-hour pretreatment of normal rats with either of the three hepatotrophic factors induced a rapid suppression of all of the tested spontaneous NK cell functions in liver-resident MNL, including direct killing of YAC-1 and P815 target cells, as well as indirect killing, such as antibody-dependent cellular cytotoxicity and NKR-P1–mediated redirected lysis of P815 target cells (Table 3). However, while ALR and IGF-II showed consistent suppressive effects in all experiments performed, as well as in a variety of assays utilized, HGF showed some inconsistency of activity, inducing in one of the experiments a stimulation of NK cytotoxicity against YAC-1 targets and a suppression of spontaneous lymphocyte-activated killing against P815 tumor cells (Table 3). In addition, combined treatment with three hepatotrophic factors showed an inconsistency of effects on liver-resident NK cells similar to HGF alone (Table 3). These data suggest that HGF may partially differ from ALR and IGF-II in mechanisms of *in vivo* induction of functional modulation of liver-resident NK cells, and that, in combined treatment, its effect is dominant.

Neither rALR, rHGF, nor rIGF-II had a significant effect on spleen or blood NK cell killing activities (data not shown).

In Vitro Effects of Hepatotrophic Factors on NK Cell Functions

To define whether hepatotrophic factor–induced changes in liver-resident NK cells are a consequence of a direct or indirect effect, we added rALR, rHGF, or rIGF-II to cultures of MNL isolated from the livers of untreated rats. The addition of the hepatotrophic factors during the 4-hour ^{51}Cr-release cytotoxicity assays did not change spontaneous NK, lymphocyte-activated killer, and antibody-dependent cellular cytotoxicity cytolytic functions or redirected killing (data not shown). Similar data were obtained when liver-resident MNL were preincubated for 24 hours in the presence of any of these hepatotrophic factors (data not shown).

DISCUSSION

Liver regeneration following a PH of 70% is associated with an increase in serum levels of ALR[1] and other growth factors, including HGF, TGF-α, and epidermal growth factors,[19,24] which peak in 12 hours and return almost to baseline by 24 hours. During approximately the same time, rapid and profound suppression of a variety of spontaneous functions of liver-resident NK cells has been shown.[21] In the present study, we showed that the treatment of normal nonoperated rats for 24 hours with exogenous rALR, rHGF, and rIGF-II caused the same liver-restricted changes in NK cell function (Table 4).

The molecules listed in Table 1 prevent *in vivo* hepatocyte atrophy as well as dramatically up-regulate hepatocyte renewal. For their full expression of a proliferative effect on hepatocytes, these hepatotrophic factors, including that of HGF and TGF-α (which also are considered primary initiators of mitosis *in vitro*[24,25]), require the prior, independent establishment of hyperplasia above resting baseline levels.[2–6,10] Recognition of the necessity for a priming stimulus underlies the delineation with the Eck fistula[2,4,10] and minimum hepatectomy models[3,5,6] used to delineate the major hepatotrophic molecules listed in Table 1. Webber, Godowski, and Fausto[26] associated the liver priming produced with minimal (30%) hepatectomy with an increased expression of a variety of proto-oncogenes, including c-*myc*.

The demonstrations that TGF-β was antihepatotrophic (causing atrophy and hypoplasia)[10,16,17] and that the serum levels of this cytokine correlated with the events of regeneration[17,25,27] raised the possibility that TGF-β was the turn-off switch that terminated liver volume restoration. The hypothesis that emerged from this collective body of work was that liver growth is regulated by an inter-reactive network of the various autocrine, paracrine, and endocrine factors.[1,19]

Another (and not mutually exclusive) theory is that organ growth is regulated by the immune system.[21,28–30] This hypothesis was strengthened when the T-cell–directed immunosuppressants, tacrolimus and cyclosporine,[11–14] which disrupt signal transduction from the T-cell receptor to the nucleus, as well as the recombinant cytosolic binding protein of tacrolimus (FKBP12),[15] were shown to be potently hepatotrophic. However, the credibility of this hypothesis lost ground when rapamycin, which also is T-cell inhibitory but by a more distal molecular site of action (proximal to the ribosomes), was shown to be potently antihepatotrophic.[18] Confusing the issue further, T-cell–deficient (nude) rats which have normal liver regeneration following PH, had augmentation of this response by tacrolimus treatment to the same extent as immunologically intact animals.[31] Because tacrolimus has no effect on the function of isolated spleen NK cells from normal[31,32] or nude rats both *in vivo* and *in vitro*,[31] the hypothesis by Itoh et al.[30] that NK cells capped the magnitude of hepatic regeneration was initially discounted by us.[31] However, in our earlier nude rat studies, the NK cell functions were determined in leukocytes isolated from peripheral lymphoid tissues (i.e., spleen), not from the liver.[31]

Our recently published investigations in normal rats following PH of 70%[21] have provided strong evidence that liver-resident (but not peripheral lymphoid organ) NK cells do in fact undergo sequential functional changes (see Table 4), and are potentially involved not only in the termination of regeneration, as postulated by Itoh et al.,[30] but also in its initiation. The *in vivo* depletion of NK cells before PH significantly augmented liver regeneration in normal rats.[21] These data and those reported herein are compatible with the hypothesis that ALR, HGF, IGF-II, and presumably other hepatotrophic factors produced at the earliest post-PH stage of liver regeneration, are associated with, and may be at least partially responsible for

the local suppression of liver-resident NK cells. This would allow conversion of the quiescent normal liver to the state of regeneration.

Interestingly, the joint administration of ALR, HGF, and IGF-II to intact animals did not produce the expected synergistic suppressive effect on liver NK cells. The modulation of NK cell functions after combined treatment was similar to that obtained with HGF alone. This finding suggests that simultaneous administration of hepatotrophic factors in optimal doses resulted in competition rather than in synergism. It is possible that, to obtain synergy, it would be necessary to use their sequential administration and different (perhaps smaller) doses than we used. Because hepatotrophic factors showed suppressive effects on NK cells only *in vivo* and only in the liver, it is conceivable that they indirectly suppress liver NK cells in intact animals by inducing the production of a suppressive factor by cells not present in the purified populations of liver-derived MNL. Congruent with this possibility, it has been shown that ALR[33] as well as IGF-II[34,35] selectively accumulate in liver sinusoids and possibly bind to the sinusoid lining (endothelial) cells, which also represent the major cells in the liver interacting with and binding liver-resident NK cells. In addition, liver sinusoid endothelial cells have been shown during post-PH liver regeneration to be early producers of TGF-β,[17] a powerful antihepatotrophic and immunosuppressive cytokine.[16,17,26] Thus, ALR as well as other hepatotrophic factors may exercise their suppressive effect on NK cells by binding to and inducing liver sinusoid endothelial cells to produce TGF-β. The implication is that hepatotrophic and antihepatotrophic factors (see Table 1) have a complex, coordinated, physiological effect.

Mesenchymal-epithelial interactions have been proposed as the essential governance mechanism of embryonal organ morphogenesis, epithelial differentiation, and tissue remodelling, of which hepatic regeneration is a prime example in mature mammals. Such speculation is supported by circumstantial evidence from tissue culture technology[36] and more recently from molecular studies of HGF (scatter factor).[37] Regeneration responses, including those following unilateral duct ligation and PH,[38] could be explained most easily by endocrine communication or, alternatively, by the migration and *in situ* activity of various hematolymphopoietic cell lineages, including NK cells.[21,39,40]

Acknowledgments

Abbreviations

PH	partial hepatectomy
HGF	hepatocyte growth factor
TGF	transforming growth factor
ALR	augmenter of liver regeneration
NK	natural killer
MNL	mononuclear leukocytes
IGF-II	insulin-like growth factor-II
Ig	immunoglobulin
NKR-P1	natural killer receptor protein 1

mAbs   monoclonal antibodies

LU   lytic unit

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Table 1

Growth Factors Revealed by Studies In Eck Fistula Models (1995)

Growth Factors	Reference
Stimulatory	
Hormones:	
Insulin	Starzl[2]
Growth Factors:	
Cytosol protein fractions or ALR	Francavilla[1,6–8]; Starzl[2,4]; LaBreque[3]; Terblanche [5]; Hagiya[9]
IGF-II	Francavilla[10]
TGF-α[*]	Francavilla[10]
HGF[*]	Francavilla[10]
Immunosuppressants	
Cyclosporine	Makowka[11]; Mazzaferro[12]
FK506	Francavilla[13]; Starzl[14]
Immunophilins	
FKBP$_{12}$	Starzl[15]
Inhibitory	
Growth factors:	
TGF-β[†]	Francavilla[16]; Braun[17]
Immunosuppressants	
Rapamycin[†]	Francavilla[18]

[*] Mitogenic in tissue culture.

[†] Inhibitory in tissue culture.

Table 2

Phenotype of Liver, Spleen, and Blood MNL Obtained From Rats Following 24-Hour Treatment With Hepatotrophic Factors

Source of MNL	CD5$^+$ (T) Cells	NKR-P1^{bright} (NK) Cells
Liver		
Vehicle (control)	25 ± 11	34 ± 9
ALR	23 ± 8	33 ± 10
IGF-II	25 ± 7	32 ± 11
HGF	28 ± 9	31 ± 8
Spleen		
Vehicle (control)	57 ± 8	8 ± 2
ALR	52 ± 5	10 ± 1
IGF-II	55 ± 4	9 ± 2
HGF	56 ± 4	11 ± 2
Blood		
Vehicle (control)	85 ± 4	6 ± 9
ALR	77 ± 6	13 ± 7
IGF-II	88 ± 7	9 ± 4
HGF	83 ± 5	12 ± 3

NOTE. Data are means ± SEM of percentages of total MNL in liver, spleen, and blood cells. Flow cytometry analysis of MNL was performed after immunostaining with fluorescein isothiocyanate–labeled anti-CD5, anti-CD3 (data not shown, similar to that obtained with anti-CD5) or anti–NKR-P1 mAbs, as described in Materials and Methods. Three animals contributed to each experiment. rALR or rIGF-II (0.5 μg) and rHGF (4.0 μg) were the total doses of the growth factors injected per individual animal during 24 hours.

Table 3

In Vivo Effects of rALR, rHGF, and rIGF-II on Cytolytic Functions of Liver-Resident NK Cells

Effectors	Targets (Cytotoxicity)	$LU_{20}/10^7NK$	% Modulation
Experiment 1			
Vehicle (Control)	YAC-1 (NK)	193.3	0
ALR		125.3	−35
HGF		140.2	−27
IGF-II		115.1	−40
Vehicle (control)	P815 (LAK)	15.4	0
ALR		9.9	−36
HGF		6.2	−60
IGF-II		4.3	−70
Vehicle (control)	P815 + αP815 Abs (ADCC)	376.0	0
ALR		261.6	−29
HGF		237.4	−35
IGF-II		138.1	−62
Vehicle (control)	P815 + αNKR-P1 mAb (redirected)	48.3	0
ALR		44.2	−9
HGF		28.6	−40
IGF-II		36.7	−24
Experiment 2			
Vehicle (control)	YAC-1 (NK)	501.6	0
ALR		333.1	−34
HGF		574.8	+15
IGF-II		418.5	−17
ALR +HGF + IGF-II		606.5	+20
Vehicle (control)	P815 (LAK)	53.8	0
ALR		26.1	−45
IGF-II		31.6	−41
HGF		37.6	−30
ALR +HGF + IGF-II		41.1	−24

NOTE. Liver-resident MNL were isolated from vehicle (control)-, rALR-, rHGF-, or rIGF-II–treated animals for 24 hours and were assessed for their cytotoxicity using various assays, as described in Materials and Methods. Data represent $LU_{20}/10^7$ NK cells calculated on the basis of the triplicate mean percentages of MNL killing in four different effector:target ratios, and the percentage of NKR-PI[bright] (NK) cells, determined by flow cytometry.[21] Results are from two experiments (n = 3 per group).

Abbreviations: LAK, lymphocyte-activated killer; ADCC, antibody-dependent cellular cytotoxicity.

Table 4

Changes of Spontaneous NK Cell Functions Following PH or Hepatotrophic Factor Treatment in Rats

	Following PH	Following Hepatotrophic Factors	
Cell Functions	3 h to 14 d	24 h *In Vivo*	*In Vitro*
YAC-1 lysis	DF	DF	NC
P815 lysis	DF	DF	NC
ADCC	DF	DF	NC
Redirected lysis	DF	DF	NC

NOTE. The summary of PH effects is from our previous publication[21] and that after treatments with hepatotrophic factors from the present study.

Abbreviations: DF, decrease of function; NC, no change of function; ADCC, antibody-dependent cellular cytotoxicity.