Coexpression of IL-6 and soluble IL-6R causes nodular regenerative hyperplasia and adenomas of the liver

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Studies with tumor necrosis factor p55 receptor- and interleukin-6 (IL-6)-deficient mice have shown that IL-6 is required for hepatocyte proliferation and reconstitution of the liver mass after partial hepatectomy. The biological activities of IL-6 are potentiated when this cytokine binds soluble forms of its specific receptor subunit (sIL-6R) and the resulting complex interacts with the transmembrane signaling chain gp130. We show here that double transgenic mice expressing high levels of both human IL-6 and sIL-6R under the control of liver-specific promoters spontaneously develop nodules of hepatocellular hyperplasia around periportal spaces and present signs of sustained hepatocyte proliferation. The resulting picture is identical to that of human nodular regenerative hyperplasia, a condition frequently associated with immunological and myeloproliferative disorders. In high expressors, hyperplastic lesions progress with time into discrete liver adenomas. These data strongly suggest that the IL-6/sIL-6R complex is both a primary stimulus to hepatocyte proliferation and a pathogenic factor of hepatocellular transformation.

Keywords: IL-6/liver adenomas/nodular hyperplasia/soluble IL-6R

Introduction

Mature hepatocytes are terminally differentiated cells that maintain the capability to undergo sustained cell proliferation and to reconstitute liver mass after partial hepatectomy or injury. Several cytokines and growth factors may participate in this process, but recent studies point to the importance of the tumor necrosis factor- α /

interleukin-6 (TNF- α /IL-6) axis, and in particular to the IL-6 cytokine as a major growth regulator in the liver after partial hepatectomy (reviewed by Michalopoulos and DeFrances, 1997).

At a very early stage of regenerative responses, TNF- α induces the synthesis of IL-6 and the rapid activation by post-translational modifications of a set of transcription factors such as STAT3, NF- κ B, AP-1 and C/EBP (Diehl *et al.*, 1994). Recent studies with TNF- α type I receptor- and IL-6-deficient mice have indeed shown that activation of this pathway is essential for the proper timing and full extent of regenerative responses (Cressman *et al.*, 1996; Yamada *et al.*, 1997); in both knock-out models, DNA synthesis after partial hepatectomy is severely impaired and activation of NF- κ B and STAT3 does not take place. However, injection of sufficient amounts of IL-6 can correct defects in DNA synthesis, hepatocyte regeneration and liver damage. Thus IL-6 is crucial to liver regenerative responses and cannot be substituted by other endogenously produced cytokines. With no hepatectomy, however, IL-6 does not provide a sufficient stimulus to promote hepatocyte proliferation, since transgenic mice over-expressing IL-6 in hepatocytes do not show any morphological liver alteration (Fattori *et al.*, 1994). In line with this finding, the direct injection of IL-6, when partial hepatectomy has not been performed, does not result in DNA synthesis (Cressman *et al.*, 1996).

The IL-6 receptor comprises two subunits with distinct functions: a specific binding subunit IL-6R (gp80), with no direct role in signaling activation; and a more promiscuous and ubiquitous receptor, gp130, which bears all the information required for the activation of the intracellular Jak-STAT signaling pathway in its intracytoplasmic region (Kishimoto *et al.*, 1995). Neither IL-6 nor IL-6R alone are able to interact with and cause homodimerization of gp130, but the complex of the two interacts with gp130 efficiently, whether the IL-6R is present as a transmembrane molecule or in soluble form (sIL-6R), devoid of its transmembrane and intracytoplasmic domains (Kishimoto *et al.*, 1995). sIL-6R therefore has the ability both to potentiate IL-6 activity in cells that express transmembrane IL-6R (Mackiewicz *et al.*, 1992) and to induce responsiveness in IL-6R⁻/gp130⁺ cells, which are otherwise cytokine-unresponsive (Hibi *et al.*, 1990). This observation is of physiological relevance because biologically active sIL-6R is released from the surface of expressing cells (Mullberg *et al.*, 1993), is found at detectable levels in body fluids (Novick *et al.*, 1989) and is often present at higher levels in a variety of diseases in association with elevated IL-6 production (Honda *et al.*, 1992; Gaillard *et al.*, 1993).

Peters *et al.* (1996) recently generated double transgenic mice expressing both human IL-6 (hIL-6) and sIL-6R in the liver parenchyma. In contrast to single transgenics,

Fig. 1. Survival curves of IL-6 transgenic (filled squares) and of IL-6/sIL-6R double transgenic mice (empty diamonds). A total of 20 single transgenics and 45 double transgenic mice were observed. The curves indicate the percentage of mice surviving at any given time.

IL-6/sIL-6R double transgenics develop progressive extra-medullary hematopoiesis in liver and spleen (Peters *et al.*, 1997b). Liver hematopoiesis involves mainly cells of the granulocytic and monocytic lineages, is initially detectable at 4–6 weeks of age and progressively increases to generate large confluent foci at the age of 16 weeks. The development of liver hematopoiesis in the double transgenics is in agreement with the recent observation that the IL-6/sIL-6R complex *in vitro* is able, in conjunction with stem cell factor, to expand early CD34⁺ progenitors that are IL-6R⁻/gp130^{low} (Sui *et al.*, 1995).

In this paper we have investigated how local overexpression of IL-6 and sIL-6R in the liver of double transgenic mice may affect hepatocyte function and proliferation. We report that elevated levels of both transgenes in hepatic periportal areas induce hepatocytes to proliferate and cause the early development of hyperplastic nodules that closely mimic human nodular regenerative hyperplasia (NRH) of the liver, a disorder characterized by diffuse micronodular transformation of the hepatic parenchyma (Stromeyer and Isak, 1981; Wanless, 1990; Moran *et al.*, 1991) and the late formation of large liver adenomas. These findings support the role of IL-6 as a major cytokine, responsible for both physiological and pathological proliferation of the hepatic parenchyma.

Results

Development of liver disease in a subset of over-expressing double IL-6/sIL-6R transgenic mice

IL-6/sIL-6R double transgenic mice were generated by intercrossing single transgenic mice in which the IL-6 and sIL-6R cDNAs were under the control of the mouse MT-I and of the rat PEPCK promoters, respectively (Fattori *et al.*, 1994; Peters *et al.*, 1996).

In IL-6 (MT-I/IL-6) mice, transgene expression is uniformly diffused throughout the liver parenchyma (Peters *et al.*, 1997a). These mice have a constantly elevated production of acute phase proteins by hepatocytes but no other signs of liver pathology (Fattori *et al.*, 1994). Although the originally established transgenic line showed poor survival due to the development of kidney myeloma following the deposition of immunoglobulin complexes in proximal kidney tubules (Fattori *et al.*, 1994), after

Fig. 2. Product of IL-6 × sIL-6R levels (both expressed in ng/ml) in IL-6/sIL-6R double transgenic mice surviving less or more than 20 weeks.

repeated backcrosses with CB7-F₁ (an intercross of Balb/c × C57Bl6) mice, animals were obtained (and used for this study) which maintained IL-6 overexpression but had a milder phenotype and an almost normal life-span (Figure 1).

Single sIL-6R mice express the transgene only in periportal areas where the PEPCK promoter is functional (Peters *et al.*, 1997a); they have a normal life span, are asymptomatic and show prolonged acute phase response after bolus injection of exogenous hIL-6, as expected from the potentiating effect of sIL-6R on IL-6 activity (Peters *et al.*, 1996).

Male MT-I/IL-6 transgenic mice, in which the hIL-6 cDNA is integrated in the X-chromosome, were bred with female homozygous for the human sIL-6R transgene; all F₁ females of this cross were double transgenic. They were born at the expected ratio, but a discrete subset (~40%) died within the first 18 weeks of life (Figure 1). As a comparison, only 10% of female MT-I/IL-6 transgenic mice died within the same period of time (Figure 1), whereas survival of female sIL-6R transgenic mice was 100% (Peters *et al.*, 1996; data not shown). A smaller group of double transgenic mice (~20% of the cohort) died between 20 and 40 weeks of age (Figure 1), the remaining 40% showed normal survival for the entire time of observation (~1.5 years). Autopsy analysis revealed diffused hemorrhages in the peritoneal cavity as well as signs of blood congestion in splenic organs. No signs of cardiac hypertrophy and/or ischemia were observed. In an attempt to correlate early death with transgene expression, we determined individual serum levels of transgene products in mice surviving less or more than 20 weeks. A striking and statistically significant correlation ($p = 0.006$) with early death was found with the product of IL-6 × sIL-6R serum levels (expressed as ng²/ml²) (Figure 2). Interestingly, double transgenics surviving >20 weeks never showed transgene product levels significantly above 5000 (with just one exception).

Double transgenics with an IL-6 × sIL-6R value >5000 had a statistically significant alteration of several biochemical parameters of liver function (Table I). In particular, albumin and total plasma protein levels were considerably lower and hyperlipidemia developed with an increase of triglycerides and cholesterol serum levels. A similar trend, albeit not statistically significant (with the

Table I. Biochemical parameters of double transgenic mice

Biochemical parameter	Mice groups		
	High IL-6×sIL-6R product (>5000)	Low IL-6×sIL-6R product (<5000)	Controls (males single transgenics)
Triglycerides (g/l)	1.89 ± 0.02 ($p = 0.117$) ^a	2.42 ± 0.94 ($p^* = 0.0002$)	0.8 ± 0.53
Cholesterol (g/l)	1.93 ± 1.77 ($p^* = 0.0046$)	0.68 ± 0.36 ($p = 0.396$)	0.82 ± 0.15
Albumin (g/l)	22.4 ± 5.1 ($p^* < 0.0001$)	34.4 ± 3.6 ($p = 0.698$)	33.8 ± 3.3
Total proteins (g/l)	41.1 ± 7.2 ($p^* < 0.0001$)	50.9 ± 5.3 ($p = 0.156$)	53.4 ± 4.4
Alkaline phosphatase (U/l)	131 ± 49 ($p = 0.145$)	166 ± 66 ($p^* = 0.0021$)	99 ± 25
Bilirubin (mg/l)	1.1 ± 0.2 ($p = 0.159$)	0.9 ± 0.4 ($p = 0.854$)	0.8 ± 0.1

^aGroup composed of two mice.

Controls are males littermates derived from the same cross, hemizygous for the sIL-6R transgene (therefore sIL-6R transgenics) but lacking the hIL-6 transgene. Deriving from the same cross, they have the same genetic background as the female double transgenic but they are asymptomatic and do not develop any pathology. Except where indicated, the groups analysed were composed of 6–7 mice in the case of high expressor double transgenics, 19–21 mice in the case of low expressor double transgenics and 7–14 mice in the case of controls (male single IL-6R transgenics). Biochemical parameters were measured as described in Materials and methods. The values of the two groups of double transgenic mice were compared with the values of the controls (males single transgenics). Asterisks indicate statistical significance ($p < 0.05$).

exception of the increase in triglycerides and alkaline phosphatase), was also observed in double transgenics with IL-6×sIL-6R values <5000 (Table I). Transaminases were not found to be elevated (not shown).

Double transgenics with greatly increased transgene products develop NRH and eventually adenomas in the liver

These observations suggested a mild hepatopathy related to the elevated expression of both transgenes, and occurring independently from the development of hematopoiesis, which does not become prominent in these animals until later (Peters *et al.*, 1997b). This was further supported by gross morphological examination of the liver in high expressors sacrificed at the age of 4–6 weeks. In 13 out of 20 cases examined, the hepatic parenchyma was almost entirely replaced by nodules varying in size from 1.2 to 4.0 mm. The nodules were yellow-tan/yellow-brown and usually visible under both capsular and cut surfaces. In order to avoid interference with extramedullary hematopoiesis, we confined liver histological analysis mainly to a number of high expressors (IL-6×sIL-6R >5000) that were sacrificed at the age of 4–6 weeks, when only scattered hematopoietic cells are found in the liver parenchyma (Peters *et al.*, 1997b).

In most cases (18/20) lobular architecture was distorted by the development of nodules of hyperplastic hepatocyte plates (Figure 3a and b). In some cases (5/18) all the nodules were smaller than the size of a lobule and were localized in the periportal region. In these livers it was also possible to identify hyperplastic areas without nodule formation or clear architectural distortion. In the remaining 13 cases, which corresponded to those mice showing evident macroscopic alterations, many nodules were larger than normal lobules. Hepatocellular hyperplasia was characterized by plates more than one cell in thickness and occasional binucleation or multinucleation of hepatocytes (Figure 3b). Nodules were surrounded by a narrow rim of atrophic hepatocytes and areas of sinusoidal dilatation, which are frequently associated with blood congestion and sometimes with small hemorrhages.

Fibrous septa between nodules were absent, while condensation of the reticulum was often present around expanding nodules (data not shown). Ultrastructural

examination of the nodules revealed that the hepatocyte plates were made up of low polarized cells with irregularly distributed intercellular junctions (Figure 4A). Hepatocyte degeneration could be detected in centrolobular regions (Figure 4B). No basement membrane was found surrounding sinusoidal endothelial cells (Figure 4C). These histological findings are similar to those observed in human NRH, a disorder characterized by diffused micronodular transformation of the hepatic parenchyma without fibrosis (Stromeyer and Isak, 1981; Wanless, 1990; Moran *et al.*, 1991).

In older double transgenics (4–6 months old), the development of extramedullary hematopoiesis in the liver was often so pronounced as to mask hepatocellular hyperplasia. We observed, however, in a small subset of animals in which hematopoiesis was less evident, the formation of large liver adenomas >1 cm in diameter that compressed the surrounding liver parenchyma. An example of these adenomas is shown in Figure 3c. Such lesions were characterized by normal appearance hepatocytes arranged in cords, sometimes thicker than normal, separated by sinusoidal vascular spaces, but without liver lobule formation. No isolated bile ducts and/or portal tracts inside the lesions were found. These lesions were not histologically distinguishable from liver adenomas previously described in TGF α transgenic mice (Jhappan *et al.*, 1990; see Discussion).

Participation of endothelial cell activation in the development of hepatocellular hyperplasia

It has been shown recently that cultured human vein endothelial cells are naturally unresponsive to IL-6 because they do not express transmembrane IL-6R. However, the combination of IL-6 + sIL-6R causes stimulation of the STAT3 signaling pathway and the expression of a subset of markers of endothelial cell activation, such as chemokines IL-8, MCP-1 and -3 and the adhesion molecule ICAM-1 (Romano *et al.*, 1997).

In order to obtain a morphological assessment of hepatic endothelia we first performed immunohistochemistry with anti-endothelial cell (anti-CD31) antibodies. This showed that sinusoids were reduced and compressed by the hepatocyte plates in the periportal region (Figure 5a and b), while sometimes appearing irregularly dilated at the periphery of

Fig. 3. Nodular hyperplasia and adenomas in the liver of IL-6/sIL-6R double transgenics. (a) The central hyperplastic nodule is surrounded by massively degenerated hepatocytes. The main pathological trait observed at this stage is the vacuolar degeneration of the normal liver parenchyma in close proximity to the nodular formation. Magnification $\times 100$. (b) The nodular formation is displacing the normal liver parenchyma and, as clearly shown, no capsular formation is detectable. Magnification $\times 200$. (c) A wide adenoma is compressing the large vessels from the contiguous periportal areas, as depicted the vessels are strongly congested. Magnification $\times 100$.

the expanding nodules. As a sign of direct activation, sinusoids stained positively with anti-laminin antibodies (Figure 5f) and their endothelial cells showed a marked expression of ICAM-1 (Figure 5d), while in control livers laminin staining was negative (Figure 5e) and ICAM-1 expression was only barely detectable (Figure 5c).

IL-6/sIL-6R double transgenics show increased hepatocyte proliferation and altered liver gene expression

The morphological development of liver hyperplasia followed by the formation of large adenomas suggested an increased proliferative activity of hepatocytes. Bromo-

Fig. 4. Electron micrographs of IL-6/sIL-6R double transgenic mouse liver. (A) A cluster of irregular hepatocytes, with altered number and distribution of cell junctions, from a nodular region. (B) Hepatocyte degeneration (endoplasmic reticulum swelling, intracytoplasmic vacuoli, mitochondrial condensation) from centrolobular regions. (C) Sinusoid dilatation and hepatocyte degeneration typical of centrolobular regions. Arrows indicate endothelial cells.

deoxyuridine (BrdU) incorporation detected by immunohistochemistry was used to measure directly the number of S-phase cells in double transgenics and in control single sIL-6R transgenics. BrdU (1 mg/ml) dissolved in drinking water and supplemented with 1% sucrose was given for 6 days to 7-week-old littermates derived from a cross between sIL-6R homozygous females and MT-I/IL-6 heterozygous males (all F₁ females of this cross were double transgenic, whereas F₁ males lacked the hIL-6 transgene and were hemizygous for the sIL-6R transgene). While single sIL-6R mice showed BrdU-labeling mainly in Kupffer cells (Figure 6a), intense hepatocyte staining was clearly detectable in double transgenics (Figure 6b

and c). At this age sustained extra-medullary hemopoiesis was detected only occasionally in double transgenic mice (Figure 6c). In order to quantitate hepatocyte proliferation at least 5000 nuclei/per liver were scored in randomly selected fields. The labeling index, expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei, ranged from 3 to 5% in sIL-6R mice (a value similar to that of control non-transgenics and of MT-I/IL-6 transgenics of the same age; not shown) and from 10 to 15% in MT-I-IL-6/sIL-6R. Quantitation of BrdU-positive hepatocytes is reported in Table II.

STAT3 is a key component of the IL-6 signaling pathway (Kishimoto *et al.*, 1995) and a principal mediator of acute phase response gene activation (Kishimoto *et al.*, 1995; Alonzi *et al.*, 1998). STAT3 is also rapidly induced in response to endogenous IL-6 production after partial hepatectomy and is believed to control the expression of a set of immediate-early genes activated during liver regeneration, including *Jun-B*, *c-fos* and *c-myc*. We assessed by Northern blot mRNA levels of STAT3, haptoglobin (HAP), as a marker of the acute phase response, and *c-myc* in double transgenic females, in male littermates hemizygous for the sIL-6R transgene (therefore sIL-6R transgenics) and in female MT-I/hIL-6 mice (high IL-6 expressors) (Figure 7). The bands were quantitated by phosphorimager and each value was normalized to the signal of β 2m (Figure 7). As sIL-6R does not have any biological activity of its own and sIL-6R transgenics are asymptomatic, the average values of these mice were assigned '1' and used as controls. For double transgenic mice, we again evaluated separately mice with an IL-6 $\times$ sIL-6R product >5000 ng²/ml² (Figure 7, lanes 8–10) and those with an IL-6 $\times$ sIL-6R product <5000 ng²/ml² (Figure 7, lanes 11–16). The results are shown in Table III. As expected, both single IL-6 and double IL-6/sIL-6R transgenics upregulated STAT3 and HAP expression, but only in the case of high expressors was overexpression statistically significant (Table III). In addition, only high expressors showed a trend to *c-myc* mRNA elevation, albeit not statistically significant (Table III). It is interesting to note that mouse 14, the animal with the most prominent upregulation of STAT3 (11-fold) and HAP (51-fold), had the highest *c-myc* upregulation (5-fold above control values).

Discussion

In this paper we show that IL-6/sIL-6R double transgenic mice which express elevated levels of both transgenes develop a diffuse micronodular transformation of the hepatic parenchyma. In a study of the same transgenics, Schirmacher *et al.* (1998) have recently reported a complex pathology constituted by hepatocellular hyperplasia, plasmacytoma formation and extramedullary hemopoiesis. Hepatocellular proliferation was surrounded by peliosis and necrosis with a concomitant elevation of transaminases. Due to the complexity of this model and in order to precisely evaluate the effect of the IL-6/sIL-6R complex on hepatocellular proliferation, we restricted histology to a subset of young transgenic animals (4–6 week old) with highly elevated levels of both transgene products. These mice did not show signs of plasmacytosis, necrotic areas were absent (Figure 3) and serum trans-

Fig. 5. Endothelial cell activation in the liver of double IL-6/sIL-6R transgenic mice. Cryostat sections of liver obtained from IL-6/sIL-6R double transgenic mice (right panels) and from normal mice (left panels) tested with anti-endothelial cell [anti-CD31; (a) and (b)], anti-ICAM-1 (c and d) and anti-laminin (e and f) antibodies. Anti-endothelial cell (anti-CD31) staining shows a reduction and compression of sinusoids by the thickened hepatocyte plates about the periportal vein in a IL-6/sIL-6R double transgenic mouse (b). Anti-ICAM-1 and anti-laminin staining show that sinusoids stain positively with anti-laminin Ab (e) and their endothelial cells reveal a marked expression of ICAM-1 (c) in IL-6/sIL-6R double transgenic mice, while in control livers laminin staining was negative (f) and ICAM-1 expression was light (d). (a–f) Original magnification $\times 200$.

aminase levels were normal. The apparent discrepancies between Schirmacher *et al.* (1988) and our current observations might be attributed in part to different selection criteria of animals to be analysed, and in part to the breeding of MT-I/IL-6 transgenics into different genetic backgrounds.

The picture we observe is virtually identical to that of patients affected by a hyperplastic condition termed nodular transformation of the liver or NRH parenchyma (Stromeyer and Isak, 1981; Wanless, 1990; Moran *et al.*, 1991). In double IL-6/sIL-6R transgenics, as in human, smaller NRH lesions are limited to the periportal regions, while the coalescence of neighboring nodules gives rise to larger lesions which sometimes contain recognizable portal tracts or central veins. A further analogy with human NRH is the lack of fibrosis.

NRH has been reported in association with autoimmune diseases such as rheumatoid arthritis and Felty's syndrome, with cryoglobulinemia, myelo or lymphoproliferative disorders, congestive cardiac failure, Budd–Chiari syndrome

and therapy with immunosuppressive agents (Lee, 1994). In human NRH, given the number of conditions associated with immune dysfunction, an immunological factor has been postulated to be responsible for hepatocyte proliferation and nodular transformation (Rougier *et al.*, 1978; Stromeyer and Isak, 1981). We propose that this factor, at least for a subset of human NRH, might be the IL-6/sIL-6R complex. This hypothesis is supported by the fact that in systemic juvenile rheumatoid arthritis, lupus erythematosus, multiple myeloma, chronic lymphocytic leukemia and B-cell non-Hodgkin's lymphoma with monoclonal gammopathy, all diseases with which NRH is associated, elevated serum IL-6/sIL-6R complexes have been found (Hirano *et al.*, 1990; Honda *et al.*, 1992; Gaillard *et al.*, 1993; De Benedetti *et al.*, 1994; Lavabre-Bertrand *et al.*, 1995; Kyrtonis *et al.*, 1996).

We therefore propose that the following series of events takes place in the liver of double transgenics. The combined expression of IL-6 and sIL-6R is a stimulus to hepatocyte proliferation, which causes nodule formation,

Fig. 6. Hepatocyte proliferation in sIL-6R single transgenics and in IL-6/sIL-6R double transgenic mice. BrdU-positive cells are shown (25×0.8 sections counterstained with hematoxylin). While in sIL-6R transgenics (a) BrdU, labeling is evident mainly in Kupffer cells, hepatocytes staining is clearly detectable in MTIL-6/sIL-6R double transgenics (b and c). Sustained hematopoiesis is evident in (c).

reduction and compression of periportal sinusoids. These sinusoidal alterations lead to a reduction in blood supply at the periphery of the hyperplastic nodules, resulting in hepatocyte atrophy associated with sinusoid dilatation and blood congestion in perinodular areas. This mechanism could explain the macroscopic nodular feature of the liver and the peritoneal hemorrhages often found at necropsy.

It has been shown recently that IL-6 in conjunction with sIL-6R renders human vein endothelial cells responsive to IL-6 (Romano *et al.*, 1997). Our findings in double transgenics confirm the ability of the IL-6/sIL-6R complex

to activate sinusoidal endothelial cells, as shown by the upregulation of ICAM-1 expression and induction of laminin. This process is not accompanied by signs of capillarization of endothelia. Endothelial activation might contribute to the development of extramedullary hematopoiesis by generating a microenvironment suitable to the homing of hematopoietic progenitors. Although activation of the sinusoid endothelium has never been carefully investigated in human NRH, it is interesting to note that extramedullary hematopoiesis has been reported in 30% of cases (Stromeyer and Isak, 1981).

The development of hepatocellular hyperplasia in double transgenics and the lack of similar findings in single IL-6 transgenics lends support to the idea that the IL-6/sIL-6R complex is a much stronger *in vivo* stimulus for hepatocytes to proliferate. This is further sustained by the observation that in double transgenics BrdU-labeling of parenchymal cells is 5- to 10-fold higher than controls. At the present time there are various possibilities as to why hyperplasia develops exclusively in high IL-6/sIL-6R producers and not in single IL-6 transgenics. First, it is possible that the IL-6 concentration (and therefore biological activity) has to overcome a certain threshold before bypassing a negative control to hepatocyte proliferation, which physiologically operates to homeostatically maintain liver mass. A second, more intriguing possibility is that only high concentrations of the IL-6/sIL-6R complex are able to stimulate a compartment of hepatocyte precursor cells located in the periportal region which do not

Table II. BrdU incorporation in the liver of double transgenic mice

Mice group	Mice number	BrdU-positive hepatocytes/mm ² of liver
Controls:	1	1.6
(male sIL-6R transgenics)	2	4.1
	3	0.8
IL-6/sIL-6R	4	7.4
(female double transgenics)	5	13.3
	6	24.4

Male MT-I/IL-6 transgenic mice, in which the human IL-6 cDNA is integrated in the X-chromosome, were bred with females homozygous for the human sIL-6R transgene; all F₁ females of this cross were double transgenic (IL-6/sIL-6R) whereas male littermates were hemizygous for the sIL-6R transgene (therefore were sIL-6R transgenics) but lacked the hIL-6 transgene (controls). BrdU was given for 6 days to 4-week-old littermates. Mice livers were fixed and BrdU incorporation was detected as described in Materials and methods.

Fig. 7. Altered liver gene expression in IL-6/sIL-6R double transgenic mice. Northern blots were performed using total liver RNA samples from control (–/–), single transgenics (hIL-6+, sIL-6+) and double transgenics (hIL-6+/sIL-6R+) and hybridized with the indicated probes. The amount of IL-6 and sIL-6 product is indicated for each double transgenic, and the level of hIL-6 expression is shown graphically. Autoradiograms were densitometrically scanned and results were normalized to the β2 microglobulin signal to determine the relative levels of STAT3, HAP and c-myc expression.

Table III. Overexpression of STAT3, HAP and c-myc in IL-6×sIL-6R double transgenic and in IL-6 transgenic mice

Mice group	Fold-overexpression as compared with sIL-6R mice		
	STAT3	HAP	c-myc
High IL-6×sIL-6R product (>5000)	6.06 (<i>p</i> * = 0.043)	29.43 (<i>p</i> * = 0.009)	2.22 (<i>p</i> = 0.26)
Low IL-6×sIL-6R product (<5000)	1.38 (<i>p</i> = 0.85)	7.06 (<i>p</i> = 0.46)	0.35 (<i>p</i> = 0.49)
hIL-6 mice	2.43 (<i>p</i> = 0.063)	10.32 (<i>p</i> = 0.29)	0.45 (<i>p</i> = 0.58)
sIL-6R mice	1.00	1.00	1.00

The bands shown in Figure 7 were quantitated by phosphorimager and each value was normalized to the signal of β2m. For each of the three mRNAs (STAT3, HAP and c-myc), the averages of values obtained for the sIL-6R mice was not significantly different from the values obtained for non-transgenic control mice (data not shown). Therefore, the average of the values of sIL-6R mice was assigned '1' and the mRNA expression levels of the other groups were normalized to that of sIL-6R mice. Probability values are shown. Asterisks indicate statistical significance (*p* < 0.05).

express transmembrane IL-6R α and have low numbers of surface gp130.

Old IL-6/sIL-6R double transgenics develop large liver adenomas. This finding is similar to previous reports on mice transgenics for TGF- α (Jhappan *et al.*, 1990), another cytokine involved in the control of hepatocyte proliferation and the maintenance of liver mass homeostasis (Derynck, 1988). Although the mechanisms responsible for the development of human hepatocellular carcinoma (HCC) have not been clearly defined, a current hypothesis is that hyperplastic responses to liver necrosis increase the risk of fixing mutagenic lesions caused in the DNA by oxidative damage occurring during chronic inflammation (Chisari, 1992). In double IL-6/sIL-6R transgenics, both proliferative and inflammatory stimuli coexist and, through the constant induction of the STAT3 pathway, may lead to activation of oncogenes as we find c-myc overexpression in at least one of the cases examined. Since IL-6 is constantly upregulated in chronic viral hepatitis and alcoholic cirrhosis (Deviere *et al.*, 1989; Napoli *et al.*, 1994; Malaguarnera *et al.*, 1997), which are the major risk factors for HCC, we suggest that this cytokine, besides playing a crucial role in controlling hepatocyte proliferation, is also involved in the pathogenesis of primary liver tumors.

Materials and methods

Transgenic mice

The generation of human sIL-6R (sIL-6R mice) and human MT-IL-6 (IL-6 mice) transgenic mice has been described previously (Fattori *et al.*, 1994; Peters *et al.*, 1996). MT-IL-6 mice were repeatedly backcrossed with CB6-F₁ mice, an intercross of Balb/c $\times$ C57Bl6. By crossing homozygous female sIL-6R mice and hemizygous male IL-6 mice (the transgene integration is in the X-chromosome), all resulting females were double transgenics and were used for the present studies.

Serum IL-6 and sIL-6R measurements

Blood was drawn from mice by retroorbital puncture. hIL-6 and human sIL-6R were quantified using the hIL-6 QuantikineTM ELISA and the human sIL-6R QuantikineTM ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Measurement of hematoclinical parameters

Triglycerides were quantified using the Unimate 5 Trig kit, cholesterol was measured using the Unimate 5 Cholesterol kit, total proteins were estimated using the Unimate 7 TP kit, alkaline phosphatase was determined using the Unimate 3 ALP kit and total bilirubin was quantified using the Unimate 3 Tbil kit, all of the above by Roche Diagnostics (Roche, Milan, Italy), following the manufacturer's instructions. Albumin was measured using a commercial albumin diagnostic kit (SGMitalia, Rome, Italy). All readings were performed by a COBAS MIRA automatic analyzer (Roche, Milan, Italy).

BrdU incorporation

Mice livers were immediately fixed in 4% formaldehyde freshly prepared from paraformaldehyde in phosphate buffer at pH 7.2 and then embedded in paraffin. Approximately 5 μ m thick serial sections were cut from each paraffin block. The sections were stained with hematoxylin eosin (HE) and treated for BrdU detection. For this purpose the sections were deparaffinized, incubated with 1% trypsin for 30 min, treated three times for 5 min each, with microwaves at 600 W power in citrate buffer at pH 6 and exposed to normal pig serum for 20 min. Then sections were incubated with a monoclonal mouse antibody against BrdU (clone BMC 9318, IgG1; Boehringer Mannheim Biochemica, Milan, Italy) for 30 min at 37°C, followed by an anti-mouse alkaline phosphatase-conjugated antibody. The site of alkaline phosphatase binding were detected using Fast Red as chromogen. Mayer hematoxylin counterstaining was performed.

Morphological analysis

Animals of 4–6 weeks of age were killed by cervical dislocation. For histologic evaluation tissue samples were fixed in 10% neutral buffered

formalin, embedded in paraffin, sectioned at 4 μ m and stained with HE, Giemsa, Masson's trichrome and Manuel's reticulum. For electron microscopy, specimens were fixed in cacodylate-buffered 2.5% glutaraldehyde, post-fixed in osmium tetroxide and then embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate–lead citrate. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with anti-endothelial cell (Mec-13.3; CD31) and anti-ELAM-1 (kindly provided by Dr A.Vecchi, Negri Nord, Milan, Italy), anti-VCAM-1 (Pharmingen, San Diego, CA), anti-ICAM-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-laminin (Becton Dickinson, Bedford, MA) antibodies (Abs). After washing, they were overlaid with biotinylated goat anti-rat, anti-hamster and anti-rabbit Ig (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ig was removed by washing, and the slides were incubated with ABC complex/AP (Dako, Glostrup, Denmark).

Northern blots

Total liver RNA was prepared, and 10 μ g per lane was electrophoresed on a 1% agarose formaldehyde gel, transferred to nitrocellulose and hybridized with nick translated probes as described (Cressman *et al.*, 1996).

Statistical analysis

A one-way ANOVA was used for statistical analyses of significance.

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