Development of Progressive Kidney Damage and Myeloma Kidney in Interleukin-6 Transgenic Mice

By E. Fattori, C. Della Rocca, P. Costa, M. Micgrio, B. Dente, L. Pozzi, and G. Ciliberto

Interleukin-6 (IL-6) is a pleiotropic cytokine that has been postulated as playing a role in the pathogenesis of multiple myeloma, chronic autoimmune diseases, and alcoholic liver cirrhosis. We generated transgenic mice carrying a fusion between the mouse metallothionein-I (MT-I) gene promoter and the human IL-6 cDNA. MT-I/IL-6 transgenics express IL-6 constitutively in the liver and secrete the cytokine in the blood. They show initially activation of acute-phase response genes and accumulation of α2- and β-globulins in the plasma, which is followed by polyclonal hypergamma-globulinemia. MT-I/IL-6 transgenics die between 12 to 20 weeks of age. Histologic examination of transgenic animals at different ages and after necropsy showed, as expected from previous studies of IL-6 disregulation in vivo, an increase in the number of megakaryocytes in the spleen and bone marrow and, at later stages, IgG plasmacytosis in the spleen, lymph nodes, and thymus. However, no plasma cell infiltration was detected in other organs. The distinguishing feature of MT-I/IL-6 transgenics is the development of a progressive kidney pathology, in which the initial membranous glomerulonephritis is followed by focal glomerulosclerosis and finally by extensive tubular damage that reproduces the damage observed in patients at terminal stages of multiple myeloma (myeloma kidney). The pathogenetic role of IL-6 overproduction and of the resulting serum protein overload in the kidney damage is discussed.

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MATERIALS AND METHODS

Generation of MT/IL-6 transgenic mice. A 639-bp cDNA coding for hIL-6 was amplified by polymerase chain reaction (PCR) and cloned into BamHI-Kpn I sites of pEMBL130. A 1.7-kb DNA fragment corresponding to the 3' portion of human β-globin gene was inserted downstream from the hIL-6 cDNA between the BamHI and the Pst I sites of the pEMBL vector to stabilize hIL-6 mRNA (Fig 1). A 1.9-kb EcoRI-Bgl II DNA fragment of the mouse metallothionein-I (MT-I) promoter was cloned upstream the hIL-6 cDNA in the EcoRV site by blunt end ligation. The Pvu II-Pvu II fragment containing the entire construct (Fig 1) was gel-purified and microinjected into the pronuclei of fertilized eggs of a cross F1(C57Bl6×DBAII).

RNA expression. RNA was isolated by the guanidine isothiocyanate method and subjected to Northern blot analysis. 32P-labeled cDNAs for hIL-6, mouse serum amyloid P, and α2 macroglobulin were used as probes.

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rat α1-acid glycoprotein,21 rat hemopexin,26 mouse albumin,27 C/EBPα,28 C/EBPβ, and CEBP/SZ9 were used as probes.

Determination of hIL-6 levels. Serum IL-6 activity was detected by the 7TD1 proliferation assay,30 in the absence or in the presence of 0.1 μg of a neutralizing monoclonal antibody (MoAb) specific for hIL-6 (MoAb8).31

Hematologic analysis, serum proteins profile, serum and urine Ig isotyping. Blood was collected from the retroorbital cavity; blood smears were stained with Hemacolour (J.T. Baker, Pittsburgh, PA) and the white blood cells (WBCs) were counted. The other hematologic parameters (red blood cells [RBCs], hemoglobin [HGB], platelets [PLT], mean platelet volume [MPV], and piastrinocrite [PCT]) were determined with the automatic analyzer Cell-Dyn 800 (Sequoia, Santa Clara, CA).

To determine the serum protein profile, 40 μL of serum was subjected to agarose electrophoresis. Strips were stained with Coomassie and the densitometric profile was determined.

Serum and urine Igs were detected by radial immunodiffusion (Binding Site Ltd, Birmingham, UK). One hundred microliters of a sera dilution and undiluted urine samples were used.

Determination of urinary proteins. Urinary proteins were measured by CombustTest RL (Boehringer, Mannheim, Germany).

Histopathology. Organs were fixed for more than 16 hours in 3.7% formaldehyde diluted in 50 mmol/L sodium phosphate buffer, pH 7.2, and then included in paraffin. Sections of 5 μm were subjected to hematoxilin-eosin staining.

Statistical analysis. Comparison of means was performed by the Student's t-test.

RESULTS

Generation of MT-I/hIL-6 transgenic mice. To drive expression of human IL-6 in the liver of transgenic mice, we used the promoter of the mouse MT-I gene.19 The construct (described in Materials and Methods and shown in Fig 1) containing the fusion gene was gel-purified as a Pvu II-Pvu II fragment and microinjected into the pronuclei of fertilized eggs from a cross F1 (C57Bl6 × DBAII). Four MT-I/hIL-6 transgenic founders were obtained. Two died at 5 and 8 weeks of age. A third one (no. 19) was sterile and was killed at 5 months of age at the first signs of illness. Histologic analysis of its tissues showed signs of IL-6–related pathology identical to those of the mice of line 28 (see below).

Only mouse 28 produced a low percentage of positive offspring (10%). Mice of line 28 die between 2 to 5 months of age, with the majority dying at 4 months (approximately 75%) (Fig 2). The onset of the disease is characterized by loss of weight and hunched posture; mice die suddenly after 3 to 4 days.

hIL-6 expression. Northern blots were performed with RNA from different tissues of mouse 19 and of F1 mice of line 28. IL-6 was expressed constitutively only in the liver of mouse 19 (data not shown) and in mice of line 28 (Fig 3). A weaker signal was detected in the heart at a slightly lower molecular weight (Fig 3). Parallel to this result, bioactive hIL-6 was detectable in the blood at a concentration of about 1 U/μL in mouse 19 and at a concentration 0.15 to 13 U/μL in mice of line 28 (Table 1). Interestingly, in the latter mice, IL-6 activity (tested in the 7TD1 proliferation assay) increased exponentially after 2 months of age (Table 1). This increase was not caused by activation of endogenous mouse IL-6 synthesis, because all bioactive IL-6 in the sera could be inhibited by a specific neutralizing antibody to human IL-6.11 (Table 1). The increase in hIL-6 blood levels with age is not caused by a progressive increase in the production of the cytokine because we could never detect the appearance of hIL-6 mRNA in organs other than the liver, or increased hIL-6 mRNA levels in the liver tissue with aging (not shown). The most likely explanation, therefore, is that the IL-6 half-life is increased either by complexation in the plasma with carrier proteins such as α2-macroglobulin,12 whose plasma levels are increased, or by impairment of kidney functions caused by kidney damage (see below).

MT-I/hIL-6 transgenic mice show chronic activation of acute-phase genes. To analyze acute-phase gene activation by IL-6 in transgenic mice, we performed Northern blots with RNA samples using several probes (Fig 4).

RNA levels for the positive acute-phase reactants hemopexin (HPX), α2-macroglobulin (α2M), α1-acid glycoprotein (AGP), and serum amyloid protein (SAP) are greatly

Fig 1. MT-I/hIL-6 construct injected into fertilized mouse eggs.

Fig 2. Survival curve of MT-I/hIL-6 transgenic mice. Plotted is the percentage of mice surviving at a given age.
increased, as well as those for transcription factors CEBP/β and CEBP/δ, which are known to be involved in the IL-6 signal transduction pathway and to control the expression of several acute-phase response genes. On the contrary, mRNA for transcription factor C/EBPα does not undergo any change in MT-I/hIL-6 transgenics and has been used as normalization standard in our Northern blots. In addition, mRNA levels for albumin, which is known to be negatively regulated during acute-phase response, were slightly decreased (Fig 4).

Biochemical and hematologic alterations. The plasma protein profile of MT-I/IL-6 mice was monitored through electrophoretic fractionation on agarose gels (Fig 5). An increase of α2- and β-globulins is already detectable in young animals, a direct consequence of acute-phase protein gene induction in the liver. In fact, α2- and β-globulin peaks comprise well-known acute-phase reactants such as α2M, haptoglobin (α2-globulins), complement C3, and HPX (β-globulins). In contrast, the increase in the α1-globulins fraction is not very pronounced, despite the high mRNA levels for AGP, a prominent α1-globulin produced by the liver. In 19-week-old transgenics, the alteration of α2- and β-fractions is accompanied by a lesser increase in the γ-globulin fraction. The most striking change in the levels of this latter fraction occurs at a later time, on average between 10 and 12 weeks. This increase becomes even more evident in clinically sick animals in which γ-globulins represent about 40% of the circulating plasma proteins. The γ-globulin peak is mainly caused by polyclonal IgG1 production, as tested by radial immunodiffusion (Fig 6).

The determination of hematologic parameters is summarized in Table 2. Transgenic animals at terminal stages develop neutrophilia and expansion of the volume of the platelets in the blood. In this case, although the number of PLT undergoes a slight and not significant increase, the MPV and PCT are both significantly higher than in nontransgenic littermates. The high level of neutrophilia is observed only in the last 3 to 4 days before death, and is not caused by a myeloproliferative disease, as has been shown in MSCV/IL-6-infected mice, but is probably consequential to kidney damage (see below). The increase of MPV can be attributed to a direct thrombopoietic activity of IL-6, which is also testified to by the higher number of megakaryocytes observed in the spleen and bone marrow (see below). The direct determination of enzymatic values in the serum (LDH, alkaline phosphatase, and transaminases) did not show any alteration (data not shown), therefore confirming that liver function was not compromised.

Plasmacytosis is restricted only to lymphoid organs in MT-I/IL-6 transgenic mice. A characteristic feature of previous models of IL-6 transgenics is the occurrence of polyclonal invasive plasmacytosis. The occurrence of IgG hypergamaglobulinemia in MT-I/IL-6 transgenics immediately suggested that a similar pathologic process was also developing in this case and would lead to death caused by massive plasma cell infiltration of the liver, kidney, heart, and lungs. To clarify this point, several animals were killed at different ages, both during the healthy period and after the onset of the disease, and their organs were analyzed. Before the onset of the disease, we detected only a progressive enlargement of the spleen, mostly of its longitudinal diameter. Other macroscopic alterations could be observed only in sick animals, which showed whitening of the kidneys and enlargement of the lymph nodes. Finally, autopsic analysis showed increased volume and clear signs of edema of the lungs.

Histologic examination of the organs and tissues at different ages showed signs of sequential and progressive pathology. In young animals, there was a detectable change in the spleen in which the picture was dominated by an elevated number of giant cells with polymorphic nuclei that could be identified as megakaryocytes (Fig 7A). The increase in megakaryocyte number is approximately threefold.

In older animals, we observed a progressive alteration of the structure of lymphoid organs, particularly the spleen and lymph nodes. In both cases there is a gradual invasion by a

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**Table 1. IL-6 Activity (7TD1 Hybridoma Assay) in the Serum of MT-I/hIL-6 Transgenics**

<table>
<thead>
<tr>
<th>MT-I/hIL-6 transgenics</th>
<th>1 mo</th>
<th>2 mos</th>
<th>3 mos</th>
<th>4 mos</th>
</tr>
</thead>
<tbody>
<tr>
<td>(U/mL)</td>
<td>0.2 ± 0.14</td>
<td>0.5 ± 0.29</td>
<td>3.0 ± 1.4</td>
<td>9.4 ± 2.9</td>
</tr>
<tr>
<td>+ MoAb8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WT littermates</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Experiments were performed with sera from at least 5 animals/time point and normalizations were performed by interpolation to a growth curve obtained with known amounts of recombinant human and mouse IL-6. In these assays, 1 U corresponded to 1 pg of recombinant mIL-6 and to 7 pg of hIL-6. Indicated are the mean values and standard deviations.
Fig 4. (A) Constitutive induction of acute-phase liver genes. Total RNA (20 μg) extracted from the liver of a 3-month-old transgenic mouse (+) and a nontransgenic littermate control (−) was subjected to Northern blot analysis. The probes used are HPX, α2M, AGP, SAP, mouse albumin cDNA (Alb), and rat CEBP/α cDNA (CEBP/α). (B) CEBP/β and CEBP/δ liver expression in the liver of a 3-month-old MT-I/IL-6 transgenic mouse (+) and of a nontransgenic littermate control (−). Northern blot analysis was performed using the filters shown in (A) after stripping of the previous probe.

A homogeneous population of compactly arranged plasmacytoid cells characterized by an eccentrically situated nucleus and a basophilic cytoplasm (compare Fig 7B [spleen] and C [peribronchial lymph node]). In cases of advanced disease, this homogeneous population totally subverts the architecture of the spleen. MT-I/IL-6 transgenics never showed major alterations in the bone marrow except for the presence of increased megakaryocytes (2-fold; data not shown). Interestingly, the same histopathologic findings, ie, an increase in splenic megakaryocytes and plasmacytosis of lymphoid organs, were also observed in founder mouse no. 19 (data not shown).

This plasmacytosis is similar, both in morphology and arrangement of the cells, to what has been previously observed in Eμ/IL-6 mice. However, surprisingly, we never observed infiltration of plasma cells in other organs such as the liver, kidney, lungs, and heart. We could conclude, therefore, that the plasmacytosis itself is not directly responsible for early death of MT-I/IL-6 transgenics.

MT-I/IL-6 transgenics develop a specific kidney pathology. Examination of transgenic kidneys at different ages and postmortem showed progressive and constant alterations that, in their final stage, testify to loss of kidney function and are responsible for the death of the animal (Fig 8).
Young animals show only an increase in the mesangial matrix of some kidney glomeruli (compare Fig 8A [normal kidney] with B [transgenic kidney]). In older animals with hypergammaglobulinemia, the number of damaged glomeruli increases and some of them show signs of glomerulosclerosis. This picture is often accompanied by the presence of protein casts in the tubuli (Fig 8C). In dead animals, the totality of the glomeruli is sclerotic and the enlargement of the tubuli is more accentuated, with deposition of proteinaceous material and tubular atrophy (Fig 8D). This is in large part caused by the precipitation of circulating Igs produced by the expanded plasma cell compartment, as indicated by direct immunostaining of kidney sections with polyclonal antimouse IgG antibodies (data not shown). This picture closely resembles that observed in patients affected by multiple myeloma. Loss of kidney function is likely to be responsible for the edema in the lungs observed during the autopsy of dead animals (Fig 9). We never observed signs of plasma cell invasion of either kidneys or lungs. Again, the same two aspects of advanced glomerulosclerosis and tubuli swelling with deposition of proteinaceous material were also observed by histology of kidneys of founder mouse no. 19 (data not shown).

To establish correlations between histopathologic findings, the development of renal insufficiency, and changes in the plasma protein profile, we examined some parameters of kidney function, such as proteinuria and blood urea nitrogen (BUN). The results are shown in Table 3. Young animals with initial thickening of the mesangial matrix show neither
Table 2. Hematologic Parameters in 3-Month-Old MT-I/hIL-6 Transgenic Mice

<table>
<thead>
<tr>
<th></th>
<th>WBC (10^6/μL)</th>
<th>Neutrophils (%)</th>
<th>RBC (10^6/μL)</th>
<th>HGB (g/dL)</th>
<th>PLT (10^9/μL)</th>
<th>MPV (μm^3)</th>
<th>PCT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>8 ± 1</td>
<td>33 ± 15</td>
<td>9.8 ± 1.5</td>
<td>17.5 ± 1</td>
<td>600 ± 100</td>
<td>6.5 ± 0.5</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>Transgenics</td>
<td>15 ± 0.5*</td>
<td>81.5 ± 1.5*</td>
<td>9.6 ± 1.5</td>
<td>15 ± 2</td>
<td>1,000 ± 300</td>
<td>11.5 ± 3.5</td>
<td>1.06 ± 0.02*</td>
</tr>
</tbody>
</table>

Indicated are the mean values and standard deviations. Analysis was performed on 10 different animals.

*p < .001.

increased BUN nor proteinuria; however, transgenics with overt signs of disease (focal glomerulosclerosis) show proteinuria, mainly caused by IgG1 (Fig 10). Moreover, in the same animals, a strong increase in BUN could be detected.

**DISCUSSION**

IL-6 gene disregulation in mice has been shown to cause various forms of pathologies of the immune and hematopoietic systems that are always fatal to the animal. To explore the pathologic consequences of IL-6 disregulation in the liver tissue, which is one of the main targets of IL-6 activity, we generated transgenic mice carrying the human IL-6 cDNA under the control of the mouse MT-I promoter. Interestingly, although these mice show all the features of acute-phase response during their entire life span, they never develop morphologic and enzymatic alterations of liver functions.

In MT-I/IL-6 transgenic mice, acute-phase response is probably caused by the establishment of an autocrine/paracrine stimulatory loop. An IL-6 autocrine loop in in vitro cultures of human hepatoma cells HepG2 leads to the loss of responsiveness to IL-6 activity. HepG2-IL-6 cells become refractory to IL-6 and lose acute-phase response, probably as a consequence of the downregulation of the specific surface receptor. Relief from this state can only be achieved by adding sufficient amounts of soluble IL-6 receptor to the cell medium, which is known to act as a potentiator of IL-6 activity. This is clearly not the case in MT-I/IL-6 transgenics, and it is thus possible to conclude that, in vivo, the liver tissue does not undergo desensitization to continuous IL-6 stimulation. This difference might be caused by differences between receptor turnover in hepatocytes and in hepatoma cells or by the presence of natural soluble IL-6 receptor in the mouse serum. More experiments will be needed to clarify this issue.

At the age of 10 to 12 weeks, MT-I/IL-6 transgenics develop an IgG plasmacytosis similar to that described for Eμ/IL-6 transgenic mice and for N2/mIL-6 mice. However, there is an important difference. In MT-I/IL-6 mice, the plasmacytosis involves only lymphoid tissues, mainly lymph nodes and spleen. Proliferating plasma cells are never detected in the blood or in other organs, such as the liver, kidney, and lungs; therefore, plasmacytosis is not by itself responsible for death.

Furthermore, the increased levels of IL-6 in circulation, although responsible for the expansion of the B-cell compartment, are not sufficient to cause the myeloproliferative
changes similar to those found in MSCV/IL-6-infected mice. The only myeloproliferative modification found in MT-I/IL-6 mice is an increase in the number of megakaryocytes in the bone marrow and spleen and a significant increase in MPV and PCT. This finding was expected on the basis of the well-known activity of IL-6 as a thrombopoietic factor. However, neither alterations in the bone marrow nor in the bone structure could be detected, thus suggesting that possible myeloproliferative changes or disregulation of bone metabolism can only occur when IL-6 overproduction takes place in the microenvironment of the bone or, in the latter case, is accompanied by estrogen deficiency.

The most interesting and distinctive feature of MT-I/IL-6 mice is kidney damage; in fact, kidney failure is responsible for death. However, it must be stressed that we never observed mesangio-proliferative glomerulonephritis. This pa-
Kidney damage in IL-6 transgenic mice

Fig 9. (A) Normal lungs of a 3-month-old mouse (original magnification × 4). (B) Lungs of a spontaneously dead 3-month-old transgenic mouse (original magnification × 4). Interstitial edema and initial fibrosis is evident.

Thology has been reported in other cases of IL-6 disregulation and has been attributed to a direct effect of IL-6 on mesangial cells. In young MT-I/hIL-6 mice, the first alteration is a general thickening of the interstitial matrix of the glomeruli. This lesion (membranous glomerulonephritis), concomitant to kidney overload by acute-phase proteins produced by the liver, is still compatible with a normal kidney function because no proteinuria is detectable in these animals. At later stages, we observed focal glomerulosclerosis and tubuli swelling as a consequence of massive deposition of protein casts. Casts are homogeneous, eosinophilic, and often accompanied by tubular atrophy. This picture is surprisingly similar to what, in human pathology, is found in patients at advanced stages of multiple myeloma known as myeloma kidney. Interestingly, we never detected signs of interstitial nephritis, a common manifestation of myeloma kidney.

Myeloma kidney is believed to be caused by massive precipitation of circulating Igs and/or the Bence-Jones protein. It is important to note that myeloma kidney in MT-I/IL-6 transgenics develops in the presence of increased polyclonal hypergammaglobulinemia, as opposed to the monoclonal hypergammaglobulinemia that characterizes human myeloma patients. However, more importantly, the same type of kidney damage is not found in Ep/IL-6 transgenics and in N2/mIL-6 mice, which have the same degree of polyclonal hypergammaglobulinemia, but apparently no acute-phase response. We therefore postulate that myeloma kidney might be the resultant of the concomitant development of sustained hypergammaglobulinemia and acute-phase response. In fact, myeloma patients show increased acute-phase response markers such as CRP and complement C3 in response to increased levels of circulating IL-6. Hence, it is possible that kidney cells cannot withstand the simultaneous overload of α2-, β-, and γ-globulins, or that a specific acute-phase protein induced by IL-6 inhibits γ-globulin resorption by tubular cells. Several studies have invoked IL-6 disregulation as the major pathogenetic event in the development of proliferative B-cell abnormalities, which are at the basis of multiple myeloma. This aspect has been investigated with success using various models of IL-6 disregulation in vivo. However, it is important to stress that only in the transgenic mice presented in this study was B-

Table 3. Parameters of Kidney Function in MT-I/hIL-6 Mice

<table>
<thead>
<tr>
<th></th>
<th>Urinary Proteins (mg/dL)</th>
<th>BUN (mg/dL)</th>
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<tr>
<td>1-2 mos of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0-15</td>
<td>41.3 ± 1.6</td>
</tr>
<tr>
<td>Transgenics</td>
<td>0-15</td>
<td>37.8 ± 3.2</td>
</tr>
<tr>
<td>3-4 mos of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0-15</td>
<td>46.9 ± 6.9</td>
</tr>
<tr>
<td>Transgenics (ill)</td>
<td>100-500</td>
<td>160.8 ± 70.3*</td>
</tr>
</tbody>
</table>

Analysis was performed on 5 different animals for each time point. Indicated are the mean values and standard deviations.

* P < .05.
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