Cloning of rat interleukin 11 and interleukin 11 receptor alpha chain and analysis of their expression in rat uterus in the peri-implantation period

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Studies in mice have shown that interleukin 11 (IL-11) signalling is required for female fertility. In the absence of IL-11, decidualization is markedly retarded and implantation fails. IL-11 acts via a heterodimeric receptor composed of a ligand-specific receptor α chain (IL-11R α) and the signalling moiety gp130. This study reports the cloning of genes encoding rat IL-11 and IL-11R α . RNase protection was used to demonstrate that expression of IL-11 is upregulated in the rat uterus at the initiation of implantation at 5.5 days after mating. Expression of the

genes encoding the two receptor components, *IL11Ra* and *gp130*, did not change throughout the peri-implantation period. *In situ* hybridization studies revealed that, as in mice, expression of IL-11 was high in the primary decidual zone at the time of the attachment reaction, whereas *IL11Ra* was expressed throughout primary and secondary decidua. Conservation of the temporal and spatial expression of IL-11 and IL-11R α in the uterus of the mouse and rat during the peri-implantation period will facilitate future studies investigating the role of IL-11 in fertility.

Introduction

Interleukin 11 (IL-11) is a cytokine that is active on a range of different types of cell. Its effects include stimulation of haematopoietic progenitors, anti-inflammatory actions, effects on bone remodelling and induction of acute phase protein secretion by the liver (Du and Williams, 1997; Schwertschlag *et al.*, 1999). Recombinant human IL-11 (rhIL-11) has been licensed as a biopharmaceutical for the treatment of post-chemotherapy-induced thrombocytopenia. Studies on a variety of animal models have indicated that rhIL-11 is efficacious in treating acute and chronic inflammation disorders, such as inflammatory bowel disease (Peterson *et al.*, 1998), hyperoxia and radiation-induced lung damage (Redlich *et al.*, 1996; Waxman *et al.*, 1999), sepsis (Barton *et al.*, 1996; Chang *et al.*, 1996; Opal *et al.*, 1998), and graft versus host disease (Hill *et al.*, 1998; Teshima *et al.*, 1999).

IL-11 is a member of a family of cytokines that includes IL-6, leukaemia inhibitory factor (LIF), oncostatin M, cardiotropin 1 and ciliary neurotrophic growth factor. Members of this cytokine family signal via a high affinity receptor complex composed of a ligand binding α subunit and a common signal transducing subunit, gp130. IL-11 binds to a complex of IL-11 receptor α (IL-11R α) and gp130. In mice, a locus encoding a second murine IL-11R α chain (*IL11Ra2*) has been identified. In contrast to the gene encoding IL-11R α (*IL11Ra*), which is expressed ubiquitously, expression of the gene encoding the second

receptor is restricted to lymphoid tissues and testis (Bilinski *et al.*, 1996; Robb *et al.*, 1996, 1997).

Mice with a null mutation of the gene encoding IL-11R α revealed a key role for IL-11 in the fertility of female mice. In IL-11R α null female mice, the uterine decidualization reaction that occurs after implantation was defective, causing embryos to die at about day 8 (Robb *et al.*, 1998). In another study, mice homozygous for a hypomorphic *IL11Ra* allele had a retarded decidualization response, in which the decidua degenerated before the formation of a chorioallantoic placenta (Bilinski *et al.*, 1998). Expression of IL-11 in the pregnant uterus was upregulated at the time of embryo implantation and *in situ* hybridization revealed high expression of IL-11 in cells of the primary decidual zone (Robb *et al.*, 1998). The presence of IL-11 mRNA (*IL-11*) and IL-11 protein in human endometrial tissues has been demonstrated. In agreement with studies in mice, immunoreactive IL-11 was at a maximum concentration in decidual cells (Dimitriadis *et al.*, 2000).

Uterine receptivity and the uterine stromal cell decidualization response are essential for the successful establishment and maintenance of pregnancy. The decidualization process, in which uterine stromal cells undergo transformation in response to the implanting blastocyst, is particularly marked in species such as rats and humans in which placentation is characterized by extensive invasion of trophoblast cells. Historically, the morphological changes that occur during decidualization have been characterized most extensively in the laboratory rat (De Feo, 1967; O'Shea *et al.*, 1983). More recently, Gu and Gibori (1995) examined the endocrine functions and gene expression profiles of rat decidual cells. The present

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study reports on cloning of cDNAs encoding rat IL-11 and IL-11R α and examines the expression of *IL-11* in the rat uterus as it undergoes decidualization in response to the implanting blastocyst.

Materials and Methods

Cloning of rat IL-11 and IL11Ra genes

A Sprague–Dawley rat testis cDNA library (Stratagene, La Jolla, CA) was screened with radiolabelled oligonucleotides for murine *IL-11* to isolate cDNAs encoding rat *IL-11*. Total RNA was prepared from rat testis using Trizol (Life Technologies, Grand Island, NY) and 5' RACE was performed using the 5' RACE Kit (Life Technologies) to obtain the 5' end of the cDNA. The nested gene-specific primers used were: 5'-TTGTGGTCTCCATCAGCTGGGAAAT-3', 5'-GAGGGTCTGAAGACACTCGAGGGG-3' and 5'-GCCAGAGGCTCAGCACCACCAGGA-3'. After nested amplification, PCR products were cloned into pGEM-T Easy (Promega, Madison, WI). The rat testis cDNA library was screened at reduced stringency with radiolabelled cDNA fragments of murine *IL11Ra* to isolate rat *IL11Ra* cDNAs. Purified cDNA clones and 5' RACE products were sequenced using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a 373A DNA sequencer (Applied Biosystems).

Northern blot analysis

Total RNA was prepared from rat tissues using Trizol (Life Technologies) and poly(A⁺) RNA was selected using oligo(dT) cellulose (Roche Diagnostics GmbH, Mannheim). Northern blot analysis was performed as described by Robb *et al.* (1996). The filters were hybridized with a [α -³²P]dATP-labelled 700 bp *IL-11* cDNA probe or with a 850 bp *IL11Ra* cDNA probe. Each filter was then stripped and rehybridized with a radiolabelled GAPDH cDNA fragment.

Collection of rat tissues

Adult (8–12 week old) female Wistar rats were mated and then killed at timed intervals by CO₂ inhalation followed by cervical dislocation for collection of the uterus. Midday of the day on which the vaginal plug was detected was considered as day 0.5 after mating. Samples for RNA preparation were frozen in liquid nitrogen and stored at -70°C before total RNA preparation. For uterine samples collected at days 2.5–4.5 after mating, an oviduct or uterine horn was flushed to ascertain the presence of fertilized eggs or blastocysts. For uterine samples collected at day 5.5 after mating, the presence of implantation sites was ascertained at the time of collection by administering 200 μ l of a 1% (w/v) solution of Chicago Sky Blue via the tail vein 5 min before the animals were killed. In addition, a portion of one uterine horn was cut into serial sections and subsequently examined using a microscope to confirm the presence of

implantation sites. All procedures were approved by the Animal Ethics Committee of the Royal Melbourne Hospital.

RNase protection analysis

RNase protection analyses were performed using the RPAIII Kit (Ambion Inc., Austin, TX) as described by Robb *et al.* (1998). Ten micrograms of RNA was used in each hybridization reaction. Two independent sets of uterine tissues from different females with a vaginal plug were collected and examined for gene expression at each of the 15 time points studied in this analysis. Results were comparable between the two sets of tissues at all time points. All cDNA fragments used as riboprobes were cloned into pBluescript SK. The rat *IL-11* riboprobes were two 257 bp (nucleotides 173–430 and nucleotides 321–578) cDNA fragments. The rat *IL11Ra* riboprobes were 242 bp (nucleotides 666–908) and 319 bp (nucleotides 845–1164) cDNA fragments. The rat LIF riboprobe was a 229 bp cDNA fragment (nucleotides 93–322, Genbank accession no. AB010275). The rat *gp130* riboprobe was a 260 bp cDNA fragment (nucleotides 1571–1830, Genbank accession no. M92340). A 316 bp fragment of the rat GAPDH cDNA (Ambion), labelled to 50-fold lower specific activity, was used to estimate RNA loading.

In situ hybridization

Tissues were fixed in 4% (w/v) paraformaldehyde and were dehydrated and embedded in paraffin wax. Adjacent sections (5 μ m in thickness) from three to six females with a vaginal plug were processed and hybridized with [α -³³P]UTP-labelled sense and antisense riboprobes as described by Lyons *et al.* (1995). Similar results were obtained in experiments using sectioned uteri from different females with vaginal plugs. Both murine and rat *IL-11* and *IL11Ra* riboprobes were used. In both cases, the murine and rat probes gave similar results, although, in general, background was lower with the rat probes.

Results

Cloning of rat genes encoding IL-11 and IL-11R α

Rat *IL-11* cDNA clones were obtained by screening a rat testis cDNA library with oligonucleotides corresponding to conserved regions of the murine *IL-11* cDNA. The 5' end of the cDNA was obtained using 5' RACE. A composite partial cDNA of 1264 nucleotides with an open reading frame of 600 nucleotides was obtained (Genbank accession no. AF34935). The deduced 199 amino acid sequence had 194 of 199 (97%) shared identity with that of the murine sequence, and 170 of 199 (86%) and 178 of 199 (90%) shared identity with non-human primate and human *IL-11* sequences, respectively (Fig. 1).

In humans, two *IL-11* transcripts of 2.5 and 1.5 kb encode the same functional protein, whereas only a single 2 kb transcript was detected in mouse RNA (Paul *et al.*, 1990;

Rat IL-11	M	N	C	V	C	R	L	V	L	V	L	S	L	W	P	D	R	V	A	P	G	P	P	A	G	S	P	R	V	S	S	D	P	R	A	D	L	D	S	A	V	L	L	T	R	S	L	L	A	D	T	R	Q																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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Fig. 1. The amino acid sequences of rat, mouse, non-human primate and human interleukin 11 (IL-11). Amino acids that are identical in all four species are shown as white lettering on black background, and amino acids conserved between mice and rats are shaded grey. The 21 amino acid leader sequence is indicated by asterisks. Genbank accession numbers are: rat, AF34935; mouse, U03421; monkey, M57766; human, M57765, X58377.

Kawashima *et al.*, 1991; Morris *et al.*, 1996). Northern blot analysis of poly(A⁺) RNA from pregnant rat uterus revealed a single transcript of approximately 2 kb (Fig. 2).

Rat *IL11Ra* cDNA was obtained by screening a cDNA library of rat testis with a murine *IL11Ra* cDNA fragment. The cDNA clones obtained all encoded a single transcript. The composite rat *IL11Ra* cDNA was 1713 nucleotides and contained an open reading frame of 1296 nucleotides (Genbank accession no. AF347936). The deduced 430 amino acid sequence was aligned with that of human and murine IL-11R α (Fig. 3). The human and rat sequences shared identity at 346 of 430 residues (80%), and the rat and murine sequences shared identity at 407 of 430 (95%) residues. Northern blot analysis revealed a single 1.8 kb *IL11Ra* transcript in all tissues examined (Fig. 2).

Expression of IL-11, IL11Ra and gp130 during the peri-implantation period

Total RNA was prepared from uteri of rats at sequential times after detection of a vaginal plug. Before implantation, the presence of blastocysts was confirmed by flushing the lumen of a portion of each specimen. Implantation sites were detected in uterine samples collected at day 5.5 after mating using the blue dye reaction (Psychoyos, 1973). RNase protection studies were performed using 10 μ g total RNA. *IL-11* transcripts were detected at a low concentration in the uteri of virgin rats and at days 0.5–4.5 after mating, whereas at day 5.5 after mating, *IL-11* expression was markedly upregulated (Fig. 4). Expression of *IL-11* remained high up to day 9.5 after mating. As in mice, expression of *LIF* preceded that of *IL-11*, and was detectable at low concentrations in the uterus of virgin rats and both before and after implantation. *LIF* expression was high in uterine samples collected at days 9.5–11.5 after mating, but decreased thereafter. Expression of

Fig. 2. Northern blot analysis of expression of genes encoding interleukin 11 (*IL-11*) and interleukin 11 receptor alpha (*IL11Ra*). Poly(A⁺) RNA prepared from rat tissues was hybridized with a radiolabelled *IL-11* or a *IL11Ra* cDNA probe. The northern blots were stripped and rehybridized with a radiolabelled cDNA fragment of rat *GAPDH* to assess RNA loading. K: kidney; L: liver; 6.5: pregnant rat uterus collected at day 6.5 after mating; 7.5: pregnant rat uterus collected at day 7.5 after mating. The positions of the 28S and 18S ribosomal bands are shown.

the two IL-11 receptor components, *IL11Ra* and *gp130*, was detected at all time points examined, although the expression of *IL11Ra* decreased after day 9.5 after mating. Similar results were obtained using different RNA samples and different *IL-11* and *IL11Ra* probes.

Expression of IL-11 in the primary decidual zone

An *in situ* hybridization analysis of uterine samples collected at days 0–11 after mating was performed using

Fig. 3. The amino acid sequences of rat, mouse and human interleukin 11 receptor alpha (IL-11R α). Amino acids that are identical in all three species are shown as white lettering on black background, and amino acids conserved between mice and rats are shaded grey. Gaps introduced to maximize the alignment are shown using dashes. Asterisks (*) indicate the potential signal sequence, crosses (+) indicate the conserved WSXWS motif and dots (●) indicate the transmembrane region. Genbank accession numbers are: rat, AF347936; mouse, U14412, X98519; human, U32324, Z38102.

[α - 33 P]UTP-labelled rat *IL-11* probes to determine the spatial expression of *IL-11* in the peri-implantation uterus. Expression of *IL-11* was first detected in uterine samples collected at day 5.5 after mating. In all sections examined, expression of *IL-11* was observed in the subepithelial primary decidual zone adjacent to the apposed blastocyst (Fig. 5). At later time points, expression of *IL-11* was detectable at a low concentration throughout decidual tissues (not shown).

Expression of IL11Ra in decidual tissue

In situ hybridization of [α - 33 P]UTP-labelled rat *IL11Ra* riboprobes to uterine sections collected at days 0–11 after mating was undertaken. Hybridization revealed a general increase in the expression of *IL11Ra* in both decidual and uterine tissues compared with adjacent sections hybridized with control sense riboprobes. In preimplantation uteri, expression of *IL11Ra* was low throughout the uterus. Day 5.5 after mating was the only time point at which a more localized pattern of expression was observed. At this time point, *IL11Ra* expression was highest in stroma adjacent to the luminal epithelium (Fig. 6a–c). Thereafter, widespread

expression of *IL11Ra* was detected throughout decidual and non-decidual tissues (Fig. 6d–f).

Discussion

The composite nucleotide sequences of rat *IL-11* and *IL-11Ra* were determined by sequencing cDNA clones obtained by screening a rat testis cDNA library. Northern blot analysis revealed a single 2 kb *IL-11* transcript in the poly(A+) RNA prepared from pregnant rat uterus. In other studies, a single 2 kb transcript was detected in RNA prepared from a murine cell line, whereas a major 2.5 and a minor 1.5 kb *IL-11* transcript were observed in several human tissues (Paul *et al.*, 1990; Kawashima *et al.*, 1991; Morris *et al.*, 1996). In humans, the two transcripts arise as a result of alternative use of two polyadenylation sites located within the 3' non-coding region of the gene (Paul *et al.*, 1990). However, in mouse and rat tissues examined to date, it appears that there is only a single *IL-11* transcript.

In the present study, cDNA clones encoding a second rat IL-11R α in a cDNA library prepared from rat testis were not obtained. Testis was the major site of expression of the

Fig. 4. RNase protection analysis of expression of genes encoding interleukin 11 (*IL-11*), leukaemia inhibitory factor (*LIF*), interleukin 11 receptor alpha (*IL11Ra*) and *gp130* in virgin and pregnant rat uterus. Ten micrograms of total RNA was used in each hybridization reaction. A *GAPDH* riboprobe, labelled to 50-fold lower specific activity, was used to quantify the amount of RNA present. Probe: undigested, full-length probe; C: probe after RNase digestion; V: virgin; 0.5–13.5: days after mating.

second IL-11R α receptor in mice (Robb *et al.*, 1997). The likely absence of the second receptor in rats is in accordance with previous observations that two, or in some cases multiple, *IL11Ra* loci arose in some inbred mouse strains as a result of a recent duplication event. Only a single locus encoding IL-11R α was found in humans (Nandurkar *et al.*, 1996), in some inbred mouse strains (Robb *et al.*, 1996) and in the wild mouse strain *Mus spretus* (L. Robb, unpublished).

Comparison of the predicted amino acid sequence of rat IL-11 with mouse, non-human primate and human IL-11 revealed that IL-11 was highly conserved among species. The 21 amino acid signal sequence was also conserved. Rat IL-11, like its human and murine equivalents, is a very basic protein, rich in proline and leucine and lacking cysteine residues and *N*-glycosylation sites. The predicted amino acid sequence of rat IL-11R α also showed a high degree of shared identity among species. The conservation of IL-11 and IL-11R α homologues is in accordance with

studies showing that rhIL-11 is active with target cells of both mice (Quesniaux *et al.*, 1993) and rats (Yonemura *et al.*, 1993).

The cloning of rat cDNAs encoding IL-11 and its receptor enabled the expression of these genes in the pregnant rat uterus to be studied by RNase protection. The levels of expression of the two receptor components, *IL11Ra* and *gp130*, were not significantly altered during the study period. *IL-11* transcripts were detected at a low concentration in the uteri of virgin rats and in rat uteri collected at days 0.5–4.5 after mating. At day 5.5 after mating, immediately after implantation, expression of *IL-11* was upregulated. Expression of *IL-11* was observed up to day 10.5 after mating, but decreased thereafter. *In situ* hybridization of uterine sections collected at day 5.5 after mating showed a marked localization of *IL-11* to the primary decidual zone. This temporal and spatial pattern of expression of *IL-11* and its receptors in the pregnant uterus was very similar to that observed in mice (Robb *et al.*, 1998). In mice, IL-11

Fig. 5. Interleukin 11 (*IL-11*) expression in the primary decidual zone at day 5.5 after mating. Serial sections of rat uterus collected at day 5.5 after mating were hybridized with sense (c,f) and antisense (a,b,d,e) rat *IL-11* riboprobes. Expression was restricted to the primary decidual zone, adjacent to the apposed blastocyst. (a,d) Light-field images; (b,c,e,f) dark-field images. em: embryo, pdz: primary decidual zone; st: uterine stroma. Scale bars represent (a–c) 50 nm and (d–f) 25 nm.

Fig. 6. Interleukin 11 receptor alpha (*IL11Ra*) expression in the pregnant rat uterus. Serial sections of rat uterus collected at day 5.5 after mating (a–c) and at day 6.5 after mating (d–f) were hybridized with sense (a,c,d,f) and antisense (b,e) rat *IL11Ra* riboprobes. At both times, expression of *IL11Ra* was higher throughout uterine and decidual tissues. (a,d) Light-field images; (b,c,e,f) dark-field images. am: antimesometrial decidua, m: mesometrial decidua, em: embryo; pdz: primary decidual zone. Scale bars represent 100 μm.

expression was regulated temporally in decidualoma (Robb *et al.*, 1998). *IL-11* expression in the decidual cells of mouse and rat uterus during the peri-implantation period indicates that the action of this cytokine may be linked to that of other implantation-specific genes induced by active blastocysts at the time of the attachment reaction. These genes include

members of the epidermal growth factor-like growth factor family, *COX-2* and *LIF* (Das *et al.*, 1994, 1995, 1997; Chakraborty *et al.*, 1996; Lim *et al.*, 1997a, 1998; Song *et al.*, 2000).

LIF expression was observed throughout the peri-implantation period, preceding the increase in *IL-11*

expression at day 5.5 after mating. LIF was detectable in the uterus from day 5.5 to day 9.5 after mating and increased at day 10.5 and day 11.5 after mating. This finding is in contrast to the pattern of *LIF* expression in mice, in which *LIF* expression is markedly upregulated at the time of implantation and decreases thereafter (Bhatt *et al.*, 1991; Shen and Leder, 1992; Robb *et al.*, 1998). In rats, the chorionallantoic placenta is established at about day 10.5 after mating. *LIF* expression has been observed in murine placenta, but not in embryos, at day 9.5–12.5 after mating (Conquet and Brulet, 1990; Bhatt *et al.*, 1991; Shen and Leder, 1992). The increase in *LIF* transcription in pregnant rat uterus at days 10.5 and 11.5 after mating may be due to expression in placenta; however, *in situ* hybridization studies would be required to confirm this contention.

Implantation relies on successful development of the embryo to the blastocyst stage and on steroid hormone priming of the endometrium to a receptive state (Cross *et al.*, 1994; Lim *et al.*, 1997b). At the site of implantation, the decidual reaction is initiated. The importance of decidualization for successful embryonic survival after implantation is well recognized. Although it has been established that decidualization is regulated, in part, by prostaglandins (Kennedy, 1994), other requirements for a normal decidual response to the implanting blastocyst are poorly understood. Results from this study have shown that, in mice, IL-11 signalling is obligatory for this process, and recent work demonstrated the localization of IL-11 immunoreactive protein in human decidual cells and its expression in these cells before the upregulation of prolactin expression (Dimitriadis *et al.*, 2000). The temporal and spatial pattern of expression of IL-11 in the rat uterus during the peri-implantation period, as demonstrated in this study, supports the notion that this cytokine is involved in decidualization. The results also validate the use of rats as a model for future studies examining the use of IL-11 receptor antagonists as contraceptive agents.

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