Increased Levels of Soluble Tumor Necrosis Factor- α Receptors in Serum from Pregnant Women and in Serum and Urine Samples from Newborns

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ABSTRACT. Secretion of soluble cytokine receptors has been suggested as a mechanism of regulating cytokine activity *in vivo*. In this study, the presence of soluble receptors for tumor necrosis factor- α (TNF- α) in serum samples from pregnant women and newborn babies was examined by using TNF- α receptor-specific immunoassays. Serum from pregnant women contained the 55-kD TNF- α receptor p55 [6.4 μ g/L, interquartile range, *i.e.* the difference between the 25th and 75th percentile, (IQR) 7.2 μ g/L], whereas p55 was not detected in control sera from nonpregnant, fertile women. The levels of p55 were also elevated in serum from newborns (3.2 μ g/L, IQR 0.7 μ g/L). Furthermore, first-voided urine from newborns contained high levels of p55 (44.6 μ g/L, IQR 96.6 μ g/L). The concentration of p55 in the neonatal period was significantly higher than that of children later in childhood ($p < 0.05$). The concentration of p55 was 7.3 μ g/L, IQR 13.8 μ g/L, in urine samples from a group of preschool children (1 mo < age < 5 y) and 6.9 μ g/L, IQR 2.9 μ g/L, in urine samples from children in a higher age group (5 y < age < 10 y). The concentration of the 75-kD TNF- α receptor in urine did not differ significantly between the different age groups studied. The elevated p55 serum levels of pregnant women and neonates are suggestive of a pregnancy-associated release of soluble receptors. The high concentration of p55 in neonatal urine may reflect a postpartum elimination of soluble receptors shed during pregnancy. Further studies are needed to determine the origin and physiologic implications of the soluble TNF- α receptors in pregnancy. (*Pediatr Res* 33: 82–87, 1993)

Abbreviations

TNF, tumor necrosis factor

TNFR, tumor necrosis factor receptor

p55, the extracellular domain of the TNFR with a molecular mass of 55 kD

p75, the extracellular domain of the TNFR with a molecular mass of 75 kD

R/p55, the ratio between the concentration of soluble p55 (μ g/L) and the concentration of protein in urine (μ g/L)

R/p75, the ratio between the concentration of soluble p75 (μ g/L) and the concentration of protein in urine (μ g/L)

IQR, the interquartile range, *i.e.* the difference between the 25th and the 75th percentile

B, biotinylated

r, recombinant

Considerable attention has been paid to the influence of cytokines in human pregnancy, and it is now well established that several cytokines have the potential to regulate growth and differentiation of trophoblasts at the fetoplacental unit in addition to the immunoactivities usually ascribed to them (1–3). TNF- α , a polypeptide cytokine produced primarily by mononuclear phagocytes (4, 5), has been detected in placenta (6–8), amniotic fluid (6), and decidua (6). Recent observations suggest that local TNF- α may contribute to the regulation of cell proliferation at the uteroplacental unit; DNA synthesis of trophoblasts is inhibited by TNF- α (9, 10). However, the detection of TNF- α at the fetoplacental unit is somewhat surprising because it is well known that TNF- α mediates effects that might be considered threatening to the well-being of the fetus; *i.e.* TNF- α is probably an important mediator in transplantation rejection (11, 12), and animal studies suggest that placental injury may be caused by TNF- α (13). Furthermore, TNF- α is apparently involved in premature pregnancy termination and abortion in mice (14, 15). In humans, elevated TNF- α levels in amniotic fluid are associated with infections and premature onset of parturition (16, 17).

The biological effects of TNF- α are mediated through its interaction with high-affinity receptors on target cells (18, 19). There are two molecular species of TNFR (20–25), the 55-kD TNFR protein, p55, and the 75-kD TNFR protein, p75. Each species also exists as soluble molecules (26, 27) that are probably shed from cells expressing TNFR on their surfaces (28), a phenomenon that has been demonstrated in activated neutrophils (29). The soluble TNFR specifically bind TNF- α (26, 30). Thus, due to their ability to compete with the cell surface receptors for TNF- α , soluble TNFR may function as inhibitors of TNF- α bioactivity (26, 30, 31). Accordingly, secretion of specific, soluble membrane receptors could serve as a mechanism for regulating cytokine activity *in vivo* (32, 33).

Previously, we have reported elevated levels of p55 in urine samples from pregnant women (34), suggesting that shedding of soluble TNFR may be a mechanism of regulating TNF- α activity in pregnancy. One purpose of the present study was to determine whether the pregnant state implies that p55 are shed into the circulation of the pregnant woman. The second purpose was to determine whether the soluble receptors are restricted to the maternal organism or whether the fetus also is exposed to the

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shed TNFR. Fetal urine influences the composition of amniotic fluid (34), and we have previously reported that amniotic fluid contains high levels of soluble TNFR (35). Accordingly, this study included an examination of the first-voided urine from newborns for the presence of TNFR. In addition, levels of p55 in neonatal serum samples were assessed.

Maternal and neonatal serum samples contained elevated levels of soluble p55, as did first-voided urine from newborn babies. Thus, the data presented in this article, together with previous observations (35), suggest that a pregnancy-associated shedding of p55 takes place. The present study suggests that the soluble receptors are distributed throughout the maternal as well as the fetal organism.

MATERIALS AND METHODS

Serum samples. Blood was obtained by venipuncture from 28 pregnant women and from 10 nonpregnant women at fertile age. All pregnant women were in spontaneous labor, and all pregnancies were considered normal. Peripheral blood was also obtained by venipuncture from 15 newborns at the Neonatal Department during the first 24 h of life. The blood was allowed to clot at room temperature for 1–3 h; after centrifugation, the serum was stored at -20°C until assayed.

Urine collection. To determine whether soluble TNFR might be released from the fetus via urine, first-voided urine was collected from 70 healthy, newborn babies (46 boys, 23 girls) delivered at term. We have previously shown that there are no systematic changes in urine TNFR concentration during a 24-h period (35), and therefore the urine samples were collected whenever obtained during the first 24-h period after delivery.

To study the TNFR elimination in the perinatal period, the concentration of soluble TNFR in urine samples from nine healthy newborns was determined on d 1, 3, and 5 after delivery. All babies were born at term, and the urine samples were collected at any time during the indicated 24 h.

First morning urine samples from 40 children at different ages were collected to obtain information about urine elimination of soluble TNFR during childhood. All children were patients at the Children's Department at the Regional Hospital during the last 4 mo of 1991. Urine samples included in the study were randomly taken from those samples brought to the laboratory for routine examination. The diagnoses of the children included in this part of the study are given in Table 1. The children were divided into three groups, each group determined by the age of the children: group A ($n = 8$), $1 \text{ d} \leq \text{age} \leq 1 \text{ mo}$; group B ($n = 22$), $1 \text{ mo} < \text{age} \leq 5 \text{ y}$; and group C ($n = 10$), $5 \text{ y} < \text{age} \leq 10 \text{ y}$ (Table 1). After collection, all urine samples were frozen at -20°C until assayed.

Informed consent was obtained from the women/mothers to collect the materials included in the study. Peripheral blood was taken from newborn babies at the Neonatal Department. For ethical reasons, neonatal serum could be obtained only if venipuncture was to be performed for other medical reasons. The study was approved by the Local Committee for Human Research.

Immunoassay for detection of soluble TNFR in urine. Immunoassays have been established for the detection of p55 and p75 in urine as described by Liabakk *et al.* (36). The immunoassays are based on pairs of MAb directed against different epitopes of p55 and p75 (21). Briefly, immunoplates were coated with MAb against either p55 (htr MAb) or p75 (utr MAb), samples were added, and bound TNFR were detected by using B htr MAb or B utr MAb, respectively (21). Bound B MAb were detected with a streptavidin-peroxidase complex (21). Absorbance was measured at 492 nm.

Urine samples were passed through an Amicon filter (Amicon Corp., Danvers, MA) with a 10-kD cutoff before addition to the immunoplates. This filtration caused a 10-fold concentration of urine samples.

Table 1. Characteristics of sick children included in study*

Case	Diagnosis	Age (mo)	p55 ($\mu\text{g/L}$)	p75 ($\mu\text{g/L}$)	Protein (mg/L)
1	Laryngitis	48.0	7.4	83.5	959
2	Bronchial asthma	24.0	34.9	41.8	748
3	Reduced appetite	20.0	19.9	10.8	259
4	Nephrotic syndrome	78.0	6.1	5.0	864
5	Lymphadenopathy	114.0	8.8	34.3	706
6	Bronchial asthma	16.0	20.3	18.9	270
7	Viral infection	84.0	6.6	20.1	528
8	Abdominal pain	72.0	9.4	24.6	586
9	Viral infection	21.0	6.9	41.5	982
10	Malabsorption	60.0	18.8	3.3	376
11	Tonsillitis	84.0	7.5	3.4	252
12	Abdominal pain	60.0	6.5	ND	161
13	Bronchial asthma	60.0	7.8	23.2	853
14	Lymphadenopathy	42.0	10.8	5.2	361
15	Inguinal hernia	3.0	6.5	33.7	788
16	Laryngitis	17.0	7.1	78.3	1298
17	Hydrothorax	0.8	19.5	5.5	234
18	Tonsillitis	96.0	6.1	1.5	40
19	Hyperbilirubinemia	0.1	98.4	74.4	946
20	Nephrotic syndrome	102.0	18.6	8.7	292
21	Neuroblastoma	42.0	29.0	10.3	352
22	RDS	0.2	46.5	53.4	829
23	RDS	0.2	53.6	45.6	599
24	BPD	3.0	4.9	14.7	742
25	Bronchial asthma	18.0	ND	20.9	893
26	Hypoglycemia	0.03	55.8	48.4	527
27	Laryngitis	72.0	3.6	101.1	1830
28	Epilepsy	12.0	7.2	52.0	540
29	Hypospadias	0.1	40.5	163.0	867
30	Viral infection	15.0	19.3	255.9	2451
31	Bronchial asthma	48.0	ND	160.9	1493
32	Intoxication	48.0	3.0	111.9	479
33	Laryngitis	4.0	5.4	2.8	458
34	Anal atresia	1.1	57.3	83.5	959
35	Tonsillitis	6.0	6.9	3.8	136
36	RDS	0.5	46.8	14.9	359
37	Fever convulsions	33.0	22.6	16.6	628
39	Diabetes mellitus	72.0	7.1	4.9	172
40	Septicemia	0.1	134.6	41.6	1340
RA	Diabetes insipidus	78.0	6.7	ND	147

*Children under study were patients from Children's Department. First-morning urine was randomly obtained, and the urine samples were examined for p55, p75, and protein. The numbers represent the measured level in urine from each individual. ND, not detected; RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia. The detection limit of the TNFR immunoassay was $0.6 \mu\text{g/L}$.

The specificity of the immunoassays was controlled by preincubation of the concentrated urine with 10 mg/L of different cytokines (human TNF- α , human TNF- β , and human interferon- γ). Only TNF- α , and to a lesser degree TNF- β , inhibited the TNFR signals from urine samples (36). These observations suggest that the immunoassays are specific for unoccupied TNFR in urine.

Immunoassay for detection of p55 in serum. The signals obtained when serum samples were added to the immunoassay described above were not inhibited when rTNF- α was added, indicating a nonspecific interaction. Thus, modifications of the immunoassay were done to assay serum samples. Immunoplates (Maxisorp, Nunc, Roskilde, Denmark) were coated with the p55-specific MAb IV 4E, which detects an epitope on p55 outside the binding of TNF- α (Lien E, Liabakk N-B, Espevik T, manuscript in preparation). The MAb was diluted in PBS, and 100 μL of antibody were added to each well in the concentration 10 mg/L. Immunoplates were incubated for 12 h at 4°C . After blocking with 0.5% BSA (RIA grade, Sigma Chemical Co., St. Louis, MO)

for 1 h at 37°C, wells were washed three times with PBS containing 0.1% Tween 20 (Sigma Chemical Co.) and 100 μ L of different dilutions of serum were added and incubated for 12 h at 4°C. Because MAb IV 4E and TNF- α bind to different epitopes of p55, bound receptor was detected by adding 50 μ g/L B rTNF- α (TNF- α -Biotin, Boehringer Mannheim, Germany) in PBS with 0.1% Tween 20 and 2% FCS (Gibco, Biocult, Glasgow, Scotland) to the wells. Immunoplates were incubated for 1 h at 37°C in humidity. After washing, bound B rTNF- α was detected with streptavidin-biotin-peroxydase complex using o-phenylenediamine as substrate (Dakopatts, Glostrup, Denmark). The reaction was stopped with 2NH₂SO₄. The absorbance was measured at 492 nm with a Titertek Multiscan Plus MKII (Flow, Boggio, Switzerland). The binding of B rTNF- α was abolished when an excess (5 mg/L) of rTNF was added to the assay, showing that the binding of B rTNF- α was not caused by an unspecific binding of biotin to the immunoplates.

The absorbance values obtained in the p55 and p75 assays for urine and serum samples were compared to the absorbance of reference standards of p55 and p75 in recombinant forms (F. Hoffmann-La Roche, Basel, Switzerland). Results are expressed in μ g/L. The detection limit for the immunoassay applied for analysis of urine samples was 0.6 μ g/L concentrated urine. The detection limit for the assay with MAb IV 4E was 0.3 μ g/L serum.

Protein concentration. To determine whether the levels of TNFR in urine were caused by changes reflected in urine protein concentration, protein concentration in all urine samples was determined. The protein concentration was determined by Biorad protein assay (Biorad Laboratories, Munich, Germany), the analysis being performed after the urine had been passed through the Amicon filter.

Statistical analysis. The distribution of TNFR in urine samples collected from the different groups of children showed a marked right-side skew with potentially high outliers. The median is therefore used as a measure of central tendency of all variables, and the variability is expressed as the IQR. TNFR concentrations in urine from different groups were compared by Kruskal-Wallis one-way analysis of variance. The Mann-Whitney test was used for comparison between pairs of groups.

p values less than 0.05 were considered statistically significant.

RESULTS

Detection of p55 in serum from pregnant women and newborn babies. The soluble 55-kD TNFR was detected in sera from all pregnant women in spontaneous labor (Fig. 1); the average p55 concentration of the pregnant group was 6.4 μ g/L, IQR 7.2 μ g/L

Fig. 1. Serum concentrations of the 55-kD TNFR in samples of peripheral blood from pregnant women in spontaneous delivery at term, from newborn babies, and from nonpregnant controls. Each data point represents the measured level in serum from one individual. The receptors were detected in an immunoassay, and the detection limit of the assay is indicated by the dotted line.

L. Serum samples from newborn babies also contained increased levels of p55 (Fig. 1). The average TNFR concentration in serum for the group of newborns was 3.2 μ g/L, IQR 0.7 μ g/L. No p55 was detected in serum samples from nonpregnant controls (Fig. 1).

Detection of p55 and p75 in urine from newborn babies. First-voided urine from newborns contained both molecular species of the TNFR at high levels (Table 2). The average concentration of p55 was 44.6 μ g/L, IQR 96.6 μ g/L, and the average concentration of p75 was 64.8 μ g/L, IQR 122.9 μ g/L, in urine samples from healthy babies delivered at term. The urine protein concentration in the newborn group was 1767 mg/L, IQR 1426 mg/L. There were no significant differences between the concentrations of p55, p75, or protein in urine samples from girls and boys (Table 2).

Changes in urine TNFR concentration during the perinatal period. Urine samples from a group of nine healthy babies, obtained on d 1, 3, and 5 after delivery, were examined to study the TNFR elimination postpartum. The concentration of p55 in urine samples on d 5 after delivery was significantly lower ($p < 0.05$) than that on d 1 and on d 3 (Table 3). However, the calculated ratios (R/p55) between the concentrations of p55 and urine protein concentrations did not differ significantly between the days studied. Thus, we conclude that the observed changes in p55 concentration may partly be caused by 1) the physiological dehydration that usually occurs during the first days postpartum; and 2) the increased fluid intake, and thus secondarily, urine dilution, which takes place approximately 5 d after delivery (37). The urine levels of p75 did not differ significantly on the days compared (Table 3); neither did the urine protein concentration (Table 3) or the ratio (R/p75) between the concentration of p75 and urine protein (numbers not shown) differ.

Changes in urine TNFR concentration during childhood. The concentration of p55 in urine was highest in the neonatal period when different age groups of children were compared (Fig. 2). Urine samples from children in group A (see Materials and Methods) contained significantly ($p < 0.01$) higher levels of p55 (50.2 μ g/L, IQR 45.8 μ g/L) than those of group B (7.3 μ g/L, IQR 13.8 μ g/L) and group C (6.9 μ g/L, IQR 2.9 μ g/L) (Fig. 2). The concentration of p75 did not differ significantly between group A (47.0 μ g/L, IQR 47.6 μ g/L), group B (22.1 μ g/L, IQR 49.6 μ g/L), or group C (6.9 μ g/L, IQR 24.1 μ g/L); neither did the protein concentration differ significantly between group A (715 mg/L, IQR 529 mg/L), group B (685 mg/L, IQR 551 mg/L)

Table 2. Concentration of soluble TNFR (p55 and p75) and protein in first-voided urine from healthy babies delivered at term*

	p55 (μ g/L)	p75 (μ g/L)	Protein (mg/L)
Boys ($n = 46$)	49.3 (106.8)	67.0 (122.9)	2015 (1452)
Girls ($n = 24$)	23.4 (41.7)	61.7 (147.2)	1719 (1540)

* Results are given as the median of the group. Numbers in parentheses express the variability as the IQR.

Table 3. Time course of concentration of soluble TNFR (p55 and p75) and protein in neonatal urine during perinatal period*

Day of study†	p55 (μ g/L)	p75 (μ g/L)	Protein (mg/L)
1‡	109.2 (160.6)	91.3 (80.1)	2557 (2454)
3	184.8 (177.9)	112.0 (56.3)	4610 (5707)
5	21.7 (41.3)	32.6 (71.0)	746 (1067)

* Results are given as the median of the group. Numbers in parentheses express the variability as the IQR.

† Urine samples were obtained at each of the given time points from nine healthy babies delivered at term.

‡ The first 24 h after delivery are defined as d 0. d 1, d 3, and d 5 indicate the 24-h periods after delivery corresponding to their number.

Fig. 2. Levels of the 55-kD TNFR (p55) in urine samples obtained from children at different ages (Table 1). The children were divided into groups determined by age: group A, 1 d < age ≤ 1 mo; group B, 1 mo < age ≤ 5 y; and group C, 5 y < age ≤ 10 y. Each data point represents the value measured in urine from one individual. The numbers of each value are given in Table 1.

L), or group C (410 mg/L, IQR 580 mg/L). Furthermore, R/p55 was higher ($p < 0.01$) in group A (9.5×10^{-5} , IQR 0.5×10^{-5}) than in group B (0.1×10^{-5} , IQR 0.4×10^{-5}) and group C (0.2×10^{-5} , IQR 0.5×10^{-5}). R/p75 was not significantly different between groups (numbers not given).

Previous work has demonstrated that urine from normal healthy adults contains no p55, as assessed in the TNFR immunoassay (35). On the other hand, p75 is normally detected in urine from adults (35), suggesting that secretion of p75 may be a normal physiologic phenomenon.

DISCUSSION

In the present study, we report detection of elevated levels of the TNF- α receptor protein p55 in serum from pregnant women at term (Fig. 1). In contrast, p55 was not detected in the serum samples from fertile, nonpregnant control women (Fig. 1). These results are in accordance with those obtained when urine samples from pregnant and nonpregnant women were examined for TNFR in a previous study; p55 was detected in urine from the pregnant group exclusively (35). Taken together, these data suggest that 1) a normal pregnancy in humans is associated with an increased release of p55 into the maternal circulation, and 2) increased levels of p55 in urine are suggestive of the concomitant presence of p55 in serum.

The presence of soluble TNFR in normal pregnancy appeared not to be restricted to the maternal organism exclusively, as neonatal serum taken during the first 24 h after delivery contained high levels of p55 (Fig. 1). The data from the neonatal serum analysis must, however, be interpreted carefully, inasmuch as the blood samples were obtained from sick, hospitalized neonates. On the other hand, high levels of p55 were also detected in first-voided urine samples from healthy newborns, supporting the conclusion that a normal human pregnancy does comprise a fetal exposure to soluble TNF- α receptors.

The urine levels of p55 were significantly higher in the neonatal period than later in childhood (Fig. 2). The variations in p55 levels between groups of children at different ages might be due to changes in renal function occurring throughout childhood. However, even when renal function was taken into consideration by calculating the ratio between p55 and the urine protein concentration (R/p55) (Table 1), urine p55 was significantly higher during the neonatal period than later in childhood. However, before conclusions can be drawn, it must be remembered that the examined neonatal samples were collected at the Neonatal Department, *i.e.* from sick children. Knowledge is sparse about the role of soluble TNFR in neonatal pathophysiology. It is well known that increased expression of TNFR on cell membranes may be induced by various inflammatory mediators, such as bacterial lipopolysaccharide (33, 38) and interferon (39), whereas

TNFR expression is down-regulated by TNF- α (40). IL-6 (41, 42), but not TNF- α (42), has been detected in raised concentrations in serum from neonates suffering from septicemia. However, no studies have demonstrated that shedding of soluble TNFR is increased by the exposure of inflammatory cytokines, and we observed no correlation between the concentration of p55 and the levels of biologically active cytokines (TNF- α , IL-1, IL-6) in amniotic fluid from women in delivery at term (unpublished experiments). To conclude, the reduced p55 levels in urine after the neonatal period compared with those of both the healthy newborn babies and the hospitalized neonates are suggestive of a postpartum renal elimination of the soluble TNFR shed during pregnancy. Further studies are needed to make final interpretations about the time course required for this neonatal elimination under nonpathologic conditions.

The present data support the conclusion that a normal human pregnancy is associated with an increased shedding of soluble TNF- α receptors (p55), which are distributed both in the maternal and the fetal organism. No conclusions can be drawn here as to whether the receptors are shed from the maternal organism, the fetus, or the placenta. Several *in vitro* studies suggest ubiquitous occurrence of membrane-associated TNFR (21, 22, 43), but sparse information is yet available about the cellular distribution of receptors for TNF- α in human tissues *in vivo*. Membrane preparations from placenta have been reported to have a high TNF- α binding capacity (44), thus suggesting that TNFR are expressed by placental cells. In a previous study, we reported the detection of an abundant expression of p55 by the villous syncytiotrophoblasts in placenta at term (45), and we have recently detected soluble TNFR in supernatants from placental cell cultures (unpublished experiments). Considered together, these observations make placental tissue a likely candidate as a cellular source for the p55 shed during pregnancy. However, the precise origin of the p55 shed during pregnancy remains an open question. Furthermore, whether the soluble TNF- α receptors influence TNF- α activity and contribute to the well being of the fetus remains to be elucidated.

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