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Transforming Growth Factor-α Expression of Renal Proximal Tubules in Wistar Rats Treated with Ferric and Aluminum Nitrilotriacetate

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A high incidence of renal adenocarcinoma has been observed in rats treated with ferric nitrilotriacetate (Fe-NTA) but not in rats treated with aluminum nitrilotriacetate (Al-NTA). Transforming growth factor (TGF)-α is one of the several cytokines that is known to be expressed in human and rat renal adenocarcinomas. However, its role in neoplastic transformation is still questionable. Therefore, we investigated the effect of repeated Fe-NTA and Al-NTA administration on renal TGF-α expression. Male Wistar rats were given Fe-NTA (n=16, 5–10 mg Fe/kg) and Al-NTA (n=19, 1–2 mg Al/kg) i.p., three times a week for 3 or 12 weeks. Another group of rats (n=4) was given Fe-NTA (5–10 mg Fe/kg) three times a week for 12 weeks and then left untreated for one year. Immunoreactivity for TGF-α was positive in the collecting ducts and on the apical surface of proximal tubules in the outer stripe of the outer medulla in all the animals including NTA-injected control animals. However, TGF-α immunoreactivity in the regenerative proximal tubular epithelium was observed only in the animals treated with Fe-NTA for 12 weeks. Northern blot analysis also showed expression of TGF-α mRNA only in animals treated with Fe-NTA for 12 weeks. The expression of TGF-α mRNA in the kidney was stronger than that in the liver or brain. TGF-α was also positive in renal cell carcinoma found in animals treated with Fe-NTA for 12 weeks and left untreated for one year. These results suggest that TGF-α expression may play an important role in renal carcinogenesis and that it may be a sensitive marker during the induction stage of renal cell carcinoma.

Key words: TGF-α — Renal cell carcinoma — Carcinogenesis — Ferric nitrilotriacetate — Aluminum nitrilotriacetate

Recently, several oncogenes and growth factors have been reported to be expressed in RCC[2].[1–4] In particular, expression of TGF-α[5,6] or the EGF-receptor[7–10] in RCC has been reported by several investigators, and it has been proposed that the autocrine mechanism constituted by TGF-α and the EGF-receptor is important for the proliferation of RCC.[11,12] It is not known whether expression of these factors is crucial for the induction of RCC. We, therefore, decided to investigate the expression of TGF-α during the induction stage of RCC using Fe-NTA. We previously demonstrated that Fe-NTA is carcinogenic in the kidneys of rats and mice.[13,14] In the present study, Al-NTA-treated rats were compared with Fe-NTA-treated rats because, although acute and subacute renal proximal tubular necrosis in rats treated with Fe-NTA[15] was histologically indistinguishable from that in rats treated with Al-NTA, renal adenocarcinoma has been induced only in Fe-NTA-treated animals.[13]

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[2] The abbreviations used are: RCC, renal cell carcinoma; NTA, nitrilotriacetic acid; Fe-NTA, ferric nitrilotriacetate; Al-NTA, aluminum nitrilotriacetate; TGF-α, transforming growth factor-α; EGF, epidermal growth factor; PCT, proximal convoluted tubular; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Animals and treatments Five-week-old male Wistar rats were purchased from Charles River Japan Inc. (Kanagawa). The animals were divided randomly into seven groups and were provided commercial rat chow (CREA, Tokyo) and tap water *ad libitum*. Fe-NTA and Al-NTA were prepared by the method described by Awai *et al.*[16] Briefly, nitrilotriacetic acid disodium salt (NTA, Nacalai Tesque, Kyoto), ferric nitrate (Wako, Osaka) and aluminum chloride (Wako) were each dissolved in Milli-Q water (Millipore-Japan, Osaka), and the Fe or Al solution was mixed with the NTA solution. The pH was adjusted to 7.0 with sodium bicarbonate (Wako). The molar ratio of Fe or Al to NTA was 1:4. Intraperitoneal administrations of Fe-NTA or Al-NTA were repeated three times a week for 3 or 12 weeks. The experimental groups and doses are summarized in Table I. The animals in groups 1–6 were killed under ether anesthesia within 48 h after the last injection. The animals of group 7 were left untreated for one year after the last injection and then killed.

Histological and immunohistochemical observation For histological observation, tissues from the left kidney were fixed with 10% phosphate-buffered formalin, embedded in paraffin, cut into 4 μm thick sections, and stained with

Table I. Experimental Groups and Doses

Group	Weeks of treatments	Treatment	Dose of Fe or Al (mg/kg)	No. of rats (No. of deaths)
1	3	NTA[a)]		8 (0)
2	3	Fe-NTA	Fe: 5–10	8 (0)
3	3	Al-NTA	Al: 1.5–2	6 (1)
4	12	NTA[a)]		6 (0)
5	12	Fe-NTA	Fe: 5–10	8 (1)
6	12	Al-NTA	Al: 1.5–2	11 (3)
7	12	Fe-NTA	Fe: 5–10	4 (0)

a) NTA dose equivalent to the NTA portion of Fe-NTA.

hematoxylin and eosin. For immunohistochemical observation, paraffin sections were stained by avidin-biotin-peroxidase methods using Vectastain ABC kits (Vector Laboratories, Burlingame, CA) and mouse monoclonal antibody which cross-reacts with human and rat TGF-α (Oncogene Science, Uniondale, NY). The primary anti-TGF-α antibody was diluted 1:400 and reacted at 4°C for 16 h. The sections were counterstained with hematoxylin. Normal mouse serum was included as a negative control. To verify that the immunoreactivity in the tissue sections was specific for TGF-α, the diluted primary antibody was absorbed for 24 h at 4°C with 5 μg/ml of human recombinant TGF-α (Oncogene Science) and EGF (Biomedical Technologies, Inc., Stoughton, MA).

Northern blot analysis Total RNA was isolated from the right kidney, liver and brain by the acid guanidinium thiocyanate-phenol-chloroform method.[17] RNA was denatured and run in 1.0% formaldehyde-agarose gels and then transferred from the gels to nitrocellulose membranes by capillary blotting. The membranes were baked at 80°C for 2 h.

The hybridization probes (NEN Research Products, Boston, MA) were: rat TGF-α oligonucleotide probe (5′-AGTGTGGGAATCTGGGCACTTGTTGAAGTG) and β-actin oligonucleotide probe (5′-GGCTGGGGT-GTTGAAGGTCTCAAACATGATCTGGGTCATC). These probes were labeled by the 5′ terminal labeling method[18] using [γ-^{32}P]ATP (Amersham International, UK) and T4 polynucleotide kinase (Takara Shuzo, Kyoto). Hybridization was performed in 6×SSC (0.9 *M* sodium chloride/90 m*M* sodium citrate)/1×Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrolidone/0.02% bovine serum albumin/0.5% SDS)/sheared and denatured salmon sperm DNA (100 mg/ml)/0.05% sodium pyrophosphate at 42°C for 16 h. The membranes were then washed at 42°C in 6×SSC/0.1% SDS for 30 min and at 60°C in 6×SSC/0.1% SDS for 1–2 min. Then the membranes were exposed to X-ray film at −70°C for 24 h.

RESULTS

Histological observations In the three-week treatment groups, atypical renal PCT cells with large nuclei, a high nucleus-to-cytoplasmic ratio, and a prominent nucleolus were observed in the animals given Fe-NTA (Fig. 1b)

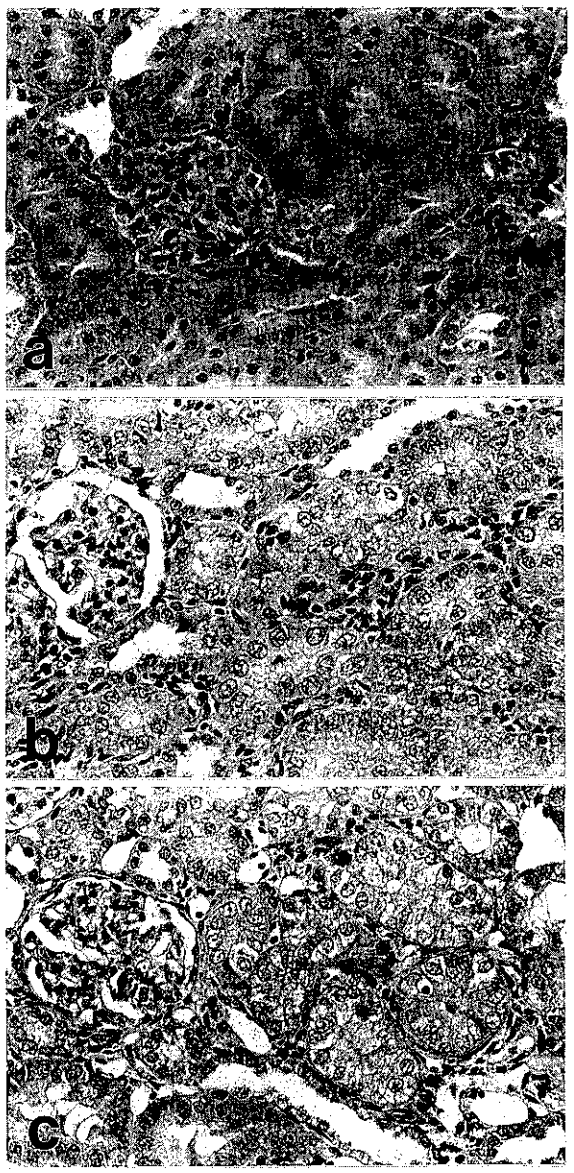


Fig. 1. Photomicrographs of kidney sections from rats treated for three weeks. (a) NTA treatment, (b) Fe-NTA treatment, (c) Al-NTA treatment. No histological change was noted in NTA-treated animal, but many atypical cells showing large nuclei, a high nuclei-to-cytoplasmic ratio, and marked nucleoli were observed in rats treated with Fe- and Al-NTA. H & E.

and Al-NTA (Fig. 1c). These were interpreted to be regenerative PCT cells. There was no histological change in the NTA-treated control animals (Fig. 1a). In the 12-week treatment groups, similar atypical PCT cells were observed in the Fe-NTA- (Fig. 2b) and Al-NTA- (Fig. 2c) treated animals but not in NTA-treated animals (Fig. 2a). Cystic dilatation of the proximal tubules was more pronounced in the 12-week-treated animals than in 3-week-treated animals. In the group which was given Fe-NTA intraperitoneally for 12 weeks and then was left for one year, RCCs were found in all four animals (Fig. 3a).

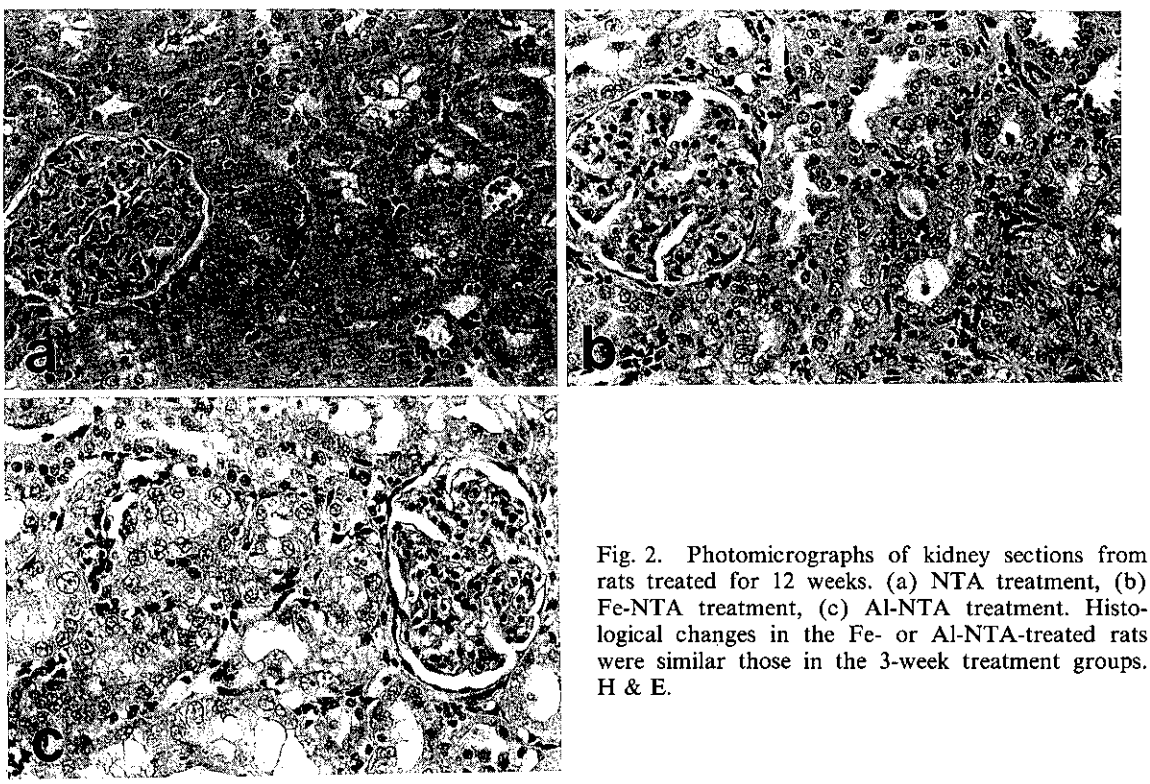


Fig. 2. Photomicrographs of kidney sections from rats treated for 12 weeks. (a) NTA treatment, (b) Fe-NTA treatment, (c) Al-NTA treatment. Histological changes in the Fe- or Al-NTA-treated rats were similar those in the 3-week treatment groups. H & E.

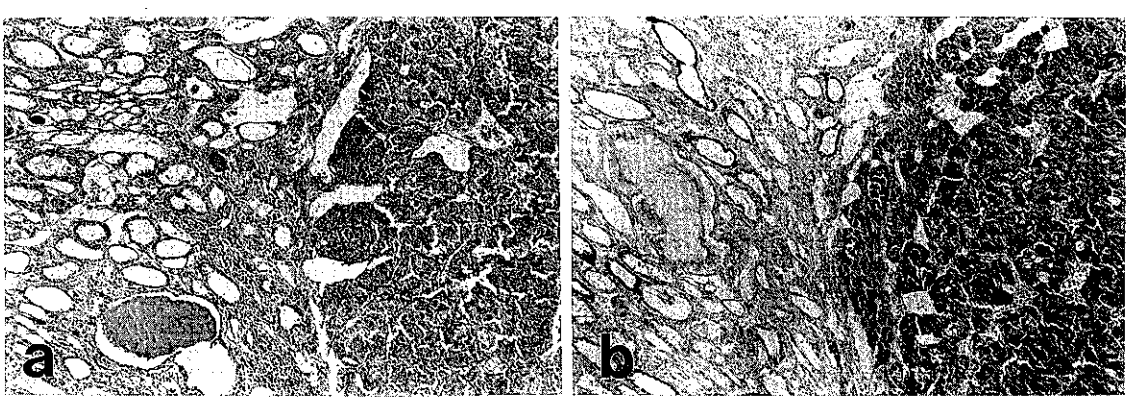
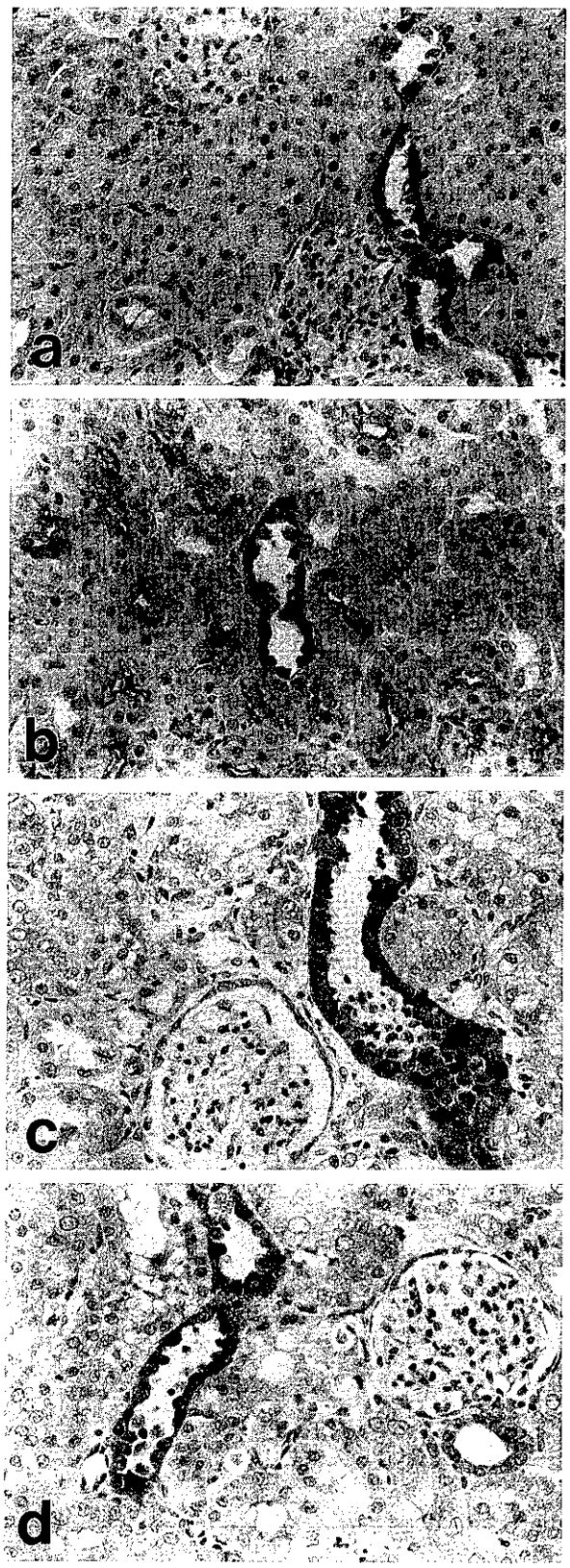


Fig. 3. Photomicrographs of RCC in an animal that was treated with Fe-NTA for 12 weeks and left for one year. (a) H & E stain, (b) immunohistochemical stain for TGF-α. In non-neoplastic tissue (left side), several collecting tubules were positive for TGF-α, and neoplastic tissue (right side) was diffusely positive for TGF-α.



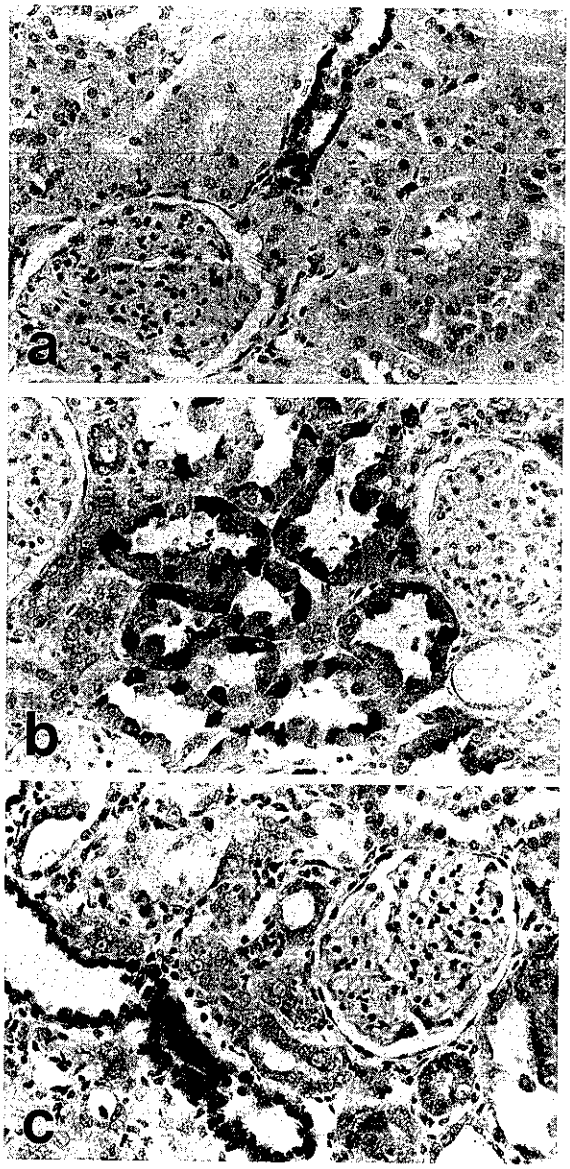


Fig. 5. Immunohistochemical staining of TGF-α on kidney sections from rats treated for 12 weeks. (a) NTA treatment, (b) Fe-NTA treatment, (c) Al-NTA treatment. Localization of TGF-α was observed in the collecting ducts in the NTA- and Al-NTA-treated animals. In the Fe-NTA-treated animals, regenerative tubules were also positive for TGF-α.

Fig. 4. Immunohistochemical staining of TGF-α on kidney sections from rats treated for three weeks. (a), (b) NTA treatment, (c) Fe-NTA treatment, (d) Al-NTA treatment. TGF-α immunoreactivity was seen in the collecting tubules (a, c, d) and on the apical surface of proximal tubules in the outer stripe of the outer medulla (b).

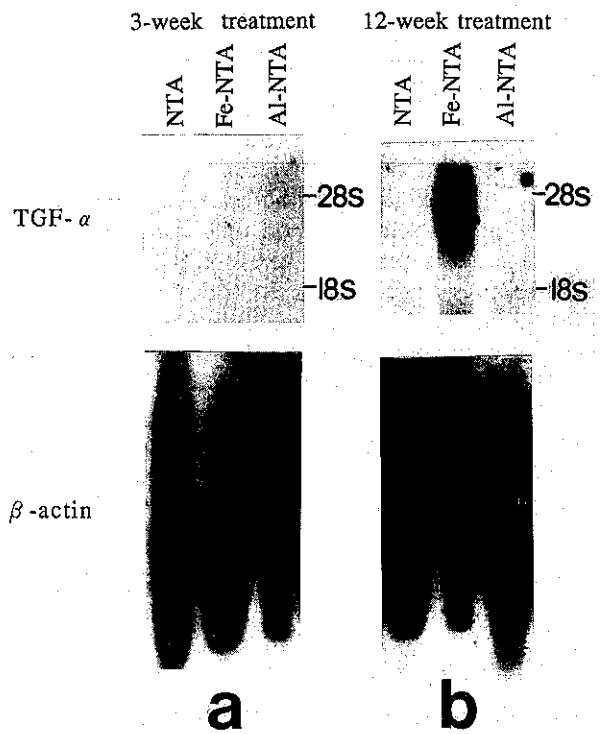


Fig. 6. RNA expression in kidney tissue. Total RNA (30 μg/lane) from the kidney tissue was examined by northern blot analysis and probed with rat TGF-α and β-actin oligonucleotides. An increased expression of TGF-α mRNA was observed in 12-week Fe-NTA-treated animals, but not in other groups.

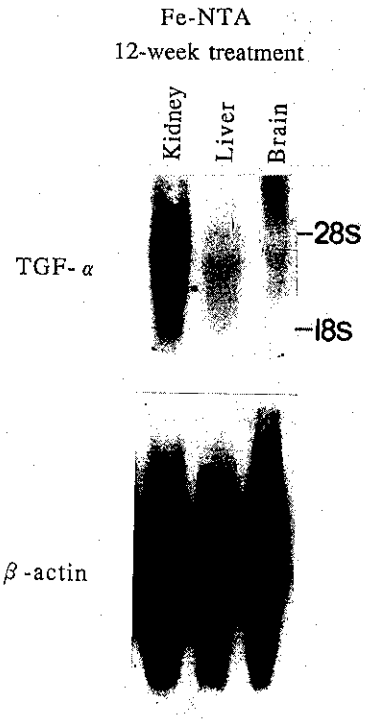


Fig. 7. RNA expression in kidney, liver and brain tissue. The total RNA (30 μg/lane) from each tissue from 12-week Fe-NTA-treated animals was examined by northern blot analysis and probed with rat TGF-α and β-actin oligo-nucleotides. The TGF-α mRNA expression in the kidney was stronger than that in the liver or brain.

Immunohistochemical observations In the three-week NTA-treated control group, the cells lining the collecting tubules were intensely positive for TGF-α (Fig. 4a). TGF-α immunoreactivity was also localized on the apical surface of the proximal tubules in the outer stripe of the outer medulla, but there was no immunoreactivity in the cytoplasm or nucleus (Fig. 4b). A similar immunoreactivity pattern was observed in the three-week Fe-NTA- (Fig. 4c) and Al-NTA-treated groups (Fig. 4d). Comparison of the groups treated with NTA or Al-NTA for 12 weeks with those treated with NTA, Fe-NTA or Al-NTA for 3 weeks showed no change in the localization of TGF-α (Figs. 5a, c). In the animals treated with Fe-NTA for 12 weeks, atypical tubules were also positive for TGF-α (Fig. 5b). The intensity of the immunoreactivity of TGF-α in the regenerative tubules was similar to that in the collecting ducts, but TGF-α-positive cells and negative cells were randomly distributed in the same tubules. In RCC induced by Fe-NTA treatment, all the neoplastic cells displayed diffuse immunoreactivity for TGF-α (Fig. 3b). Immunoreactivity was abolished by absorption of the anti-TGF-α antibody with TGF-α but not EGF (data not shown).

Northern blot analysis There was no expression of TGF-α mRNA in the kidney of the 3-week treatment groups (Fig. 6a). In the 12-week treatment groups, an increased expression of renal TGF-α mRNA was observed in the Fe-NTA-treated animals but not in the NTA- or Al-NTA-treated animals (Fig. 6b). In the 12-week Fe-NTA treatment group, the expression of TGF-α mRNA in the kidney was stronger than that in the liver or brain (Fig. 7). Northern blot analysis was not performed on the animals in which RCC was induced by Fe-NTA.

DISCUSSION

TGF-α itself has transformation activity *in vitro*[19] and overexpression of human TGF-α in transgenic mice has been shown to induce hepatocellular carcinoma and mammary adenocarcinoma.[20, 21] In addition, it is known that TGF-α is expressed in human and rat RCC.[5, 6, 11, 12]

In this study, Fe-NTA treatment for 12 weeks induced TGF-α expression in atypical regenerative PCT cells. This Fe-NTA treatment for 12 weeks is enough to induce renal adenocarcinoma.[13, 14] In animals treated with Al-NTA for 3 or 12 weeks or with Fe-NTA for 3 weeks, many regenerative PCT cells were observed in the kidney. However, there was no expression of TGF-α immunoreactivity or mRNA in the regenerative PCT cells or northern blot analysis. Therefore, the increased expression of TGF-α observed in the 12-week Fe-NTA treatment group does not appear to be simply a reflection of the increased proliferation capacity of the regenerative epithelial cells. In this regard, the immunoreactivity for proliferating cell nuclear antigen did not differ very much from that in animals treated with Fe-NTA or Al-NTA (personal observation). Therefore, the results of the present study might indicate that TGF-α plays some role in the renal carcinogenesis induced by Fe-NTA. Our immunohistochemical observations of regenerative PCT cells stained for TGF-α may support the hypothesis that RCC originates from PCT cells.[22]

The interaction between TGF-α and the EGF-receptor may play a role in promoting transformation and/or proliferation of kidney neoplasms by an autocrine mechanism.[11, 12] As TGF-α itself stimulates expression of the EGF-receptor,[12] an autocrine mechanism may function in regenerative epithelial cells following Fe-NTA treatment for 12 weeks. Since gene amplification of TGF-α and the EGF-receptor was not observed in human RCC by southern blot analysis,[5] abnormal regulation of transcription from DNA to RNA must influence the expression of TGF-α and the EGF-receptor.

Rearrangement or loss of chromosome 3p has been generally observed in human hereditary and spontaneous RCC[23–27] and a deleted region of chromosome 3p has been examined in detail.[28] Therefore, it is proposed that cancer suppressor gene(s) for RCC exist on chromosome 3p. In hereditary rat RCC, monosomy of chromosomes 5 and 6 has commonly been found[29] and similar chromosomal changes have been noted in a chemically transformed rat renal epithelial cell line *in vitro*.[30] Suppressor gene(s) of rat RCC may exist on chromosomes 5 and 6 and may be related to renal carcinogenicity.

The present results are consistent with the hypothesis that RCC originates from the renal proximal tubules and that the expression of TGF-α is important in the transition to neoplasia.

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