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Chikako Kakehashi* Masaharu Mori†
Teruyuki Kawabata‡ Shigeru Okada**

*Okayama Prefectural University,
†Okayama Prefectural University,
‡Okayama University,
**Okayama University,

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## Abstract

Administration of ferric nitrilotriacetate (Fe-NTA) in vivo causes acute renal tubular injury and finally induces renal cell carcinoma. There is accumulating evidence that these processes involve free radicals generated by Fe-NTA. To study the mechanism of renal carcinogenesis by Fe-NTA, we attempted to induce malignant transformation of primary cultured renal cells by treatment with Fe-NTA. When primary cultured renal cells (PRC) were treated continuously with Fe-NTA, all of the PRC died without transformation. On the other hand, when PRC were treated intermittently with Fe-NTA, transformed epithelial colonies were observed at 3 weeks after the first treatment. The established transformed cell line (RK523) showed drastic morphological transformation, grew in soft agar, and formed tumors when transplanted into athymic nude mice. These results indicate that the balance between cytotoxicity and mutagenecity is important for Fe-NTA induced transformation. The RK523 cell line may be a useful model for studying renal carcinogenesis in vitro.

**KEYWORDS:** renal cell, Fe-NTA(ferric nitrilotriacetate), malignant transformation, in vitro

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*Original Article*

# *In vitro* Transformation of Rat Renal Cells by Treatment with Ferric Nitrilotriacetate

**Chikako Kakehashi[a], Masaharu Mori[a]*, Teruyuki Kawabata[b], and Shigeru Okada[b]**

[a]*Department of Nursing, Faculty of Health and Welfare Science, Okayama Prefectural University, Soja 719-1197, Japan, and*
[b]*Department of Pathology, Okayama University Medical School, Okayama 700-8558, Japan*

**Administration of ferric nitrilotriacetate (Fe-NTA) *in vivo* causes acute renal tubular injury and finally induces renal cell carcinoma. There is accumulating evidence that these processes involve free radicals generated by Fe-NTA. To study the mechanism of renal carcinogenesis by Fe-NTA, we attempted to induce malignant transformation of primary cultured renal cells by treatment with Fe-NTA. When primary cultured renal cells (PRC) were treated continuously with Fe-NTA, all of the PRC died without transformation. On the other hand, when PRC were treated intermittently with Fe-NTA, transformed epithelial colonies were observed at 3 weeks after the first treatment. The established transformed cell line (RK523) showed drastic morphological transformation, grew in soft agar, and formed tumors when transplanted into athymic nude mice. These results indicate that the balance between cytotoxicity and mutagenecity is important for Fe-NTA induced transformation. The RK523 cell line may be a useful model for studying renal carcinogenesis *in vitro*.**

**Key words:** renal cell, Fe-NTA (ferric nitrilotriacetate), malignant transformation, *in vitro*

Iron is an important element that modulates the production of reactive oxygen species, which are thought to play a causative role in biological processes such as apoptosis, mutagenesis, carcinogenesis, reproductive cell death, and aging [1-3]. In hemochromatosis, primary iron-overload in humans, iron deposits in parenchymal cells such as hepatocytes, heart muscle cells, and endocrine cells of the Langerhans' islet, causes functional damage to those organs, and finally hepatocellular carcinoma develops [4, 5].

Awai *et al.* reported a model of iron overload in rats by multiple injection of an iron-chelate compound, ferric-nitrilotriacetate (Fe-NTA) [6]. They observed glucosuria and heavy deposition of iron in the liver and pancreas; features similar to those of hemochromatosis. Okada and Midorikawa later reported on the development of renal cell carcinomas in rats treated with Fe-NTA [7]. They also reported on sex difference in the incidence of tumors [8], and the effects of sex hormones [9, 10] and anti-oxidants [11, 12] on Fe-NTA-induced nephrotoxicity. A great deal of evidence has shown that the effects of Fe-NTA result from free radicals generated by Fe-NTA [13]. These free radicals damage cells by lipid peroxidation and production of 8-hydroxydeoxyguanosine [13-15]. Toyokuni and Sagripanti suggested that the carcinogenicity of Fe-NTA is also related to its ability to form oxygen free radicals in renal tubules [16].

The results of an *in vitro* study on Fe-NTA also showed production of free radicals, lipid peroxidation, mutagenecity, and cytotoxicity [17-19]. One report on

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*Corresponding author. Phone: +81-866-94-2174; Fax: +81-866-94-2174
E-mail: mmori@fhw.oka-pu.ac.jp (M. Mori)

the tumorigenecity of Fe-NTA *in vitro* was conducted (Yamada *et al.* 1990) on the transformation of the rat liver cell line RL34 [20]. To our knowledge, no other reports on *in vitro* transformation of culture cells by Fe-NTA administration have appeared. Nakatsuka *et al.* reported on the mutagenic effect of Fe-NTA on V79 Chinese hamster cells, but did not find malignant transformation [21]. To study renal carcinogenesis *in vitro,* we attempted to induce malignant transformation of primary cultured renal cells and to establish a transformed cell line by treatment with Fe-NTA *in vitro.*

## Materials and Methods

***Preparation of Fe-NTA solution.*** An Fe-NTA solution was prepared according to the method described by Awai *et al* [6]. In brief, ferric nitrate (Wako, Osaka, Japan) was dissolved in 1 N HCl solution, and nitrilotriacetic acid disodium salt (Nakarai, Kyoto, Japan) was dissolved in distilled water. These solutions were mixed with a 4-fold molar excess of nitrilotriacetic acid disodium salt, and the pH was adjusted to 7.4 by adding sodium bicarbonate (Wako) with magnetic stirring. The Fe-NTA solution was prepared immediately before use, then sterilized by Millipore (0.22 $\mu$m) filtration.

***Primary cultured renal cells (PRC).*** Six-week old male Wistar rats (Clea, Tokyo, Japan) were sacrificed under ether anesthesia. Kidneys were removed and minced in phosphate-buffered saline (PBS). They were then digested with 0.25% collagenase (Sankojunyaku, Tokyo, Japan) at 37 °C for 30 min and washed twice with Iscove's modified Dulbecco's medium (IMDM) (Gibco, NY, USA). The superficial white layer of sediment, containing mainly fragments of tubules and some glomeruli, was collected, suspended in IMDM with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), then seeded in collagen-coated 35 mm Petri dishes (Corning, Chiba, Japan). The cells were incubated in a humidified incubator at 37 °C in an atmosphere of 5% $CO_2$ in air. One day after inoculation, the medium was changed in order to discard the unattached fragments. Eight days after inoculation, when the cells had reached confluence, they were dispersed with 0.025% pronase (Boehlinger, Mannheim, Germany) with 0.2 mM EDTA (pronase-EDTA), and seeded at a density of $1 \times 10^5$ cells/well in collagen-coated 6-well dishes (Corning) with 2 ml of IMDM + 10% FBS.

***Treatment with Fe-NTA.*** Three days after inoculation, when the cells had reached confluence, they were treated as follows.

1. Fe-NTA-continuously-treated group: We performed 2 series of experiments with various concentrations of Fe-NTA; one with 0.1, 0.5, 2, 10, and 50 $\mu$g iron/ml of Fe-NTA, and the other with 0.5, 1, 2, 5, 10, 20, and 50 $\mu$g iron/ml of Fe-NTA. Cells were cultured with each concentration of Fe-NTA continuously in IMDM + 10% FBS. The medium was refreshed twice a week for up to 4 weeks. Two wells were used for each concentration of Fe-NTA. Cells cultured with Na-NTA instead of Fe-NTA were used as a control.

2. Fe-NTA-intermittently-treated group: A total of 10 series of intermittent treatments with Fe-NTA were performed; one with 0.5, 1, 2, 5, and 10 $\mu$g iron/ml of Fe-NTA 3 times a week, and 9 with 5, 10, 20, and 50 $\mu$g iron/ml of Fe-NTA, once, twice, or 3-times treatments a week. Cells were cultured in media containing various concentrations of Fe-NTA for 6 h, then each medium was substituted with fresh IMDM + 10% FBS. The same treatment was repeated for up to 4 weeks. Two wells were used for each concentration of Fe-NTA. Cells cultured with Na-NTA instead of Fe-NTA were used as a control.

***Estimation of cell growth.*** For estimation of cell growth rate, cells were seeded at a density of $1 \times 10^5$ cells in a 25 $cm^2$ collagen-coated culture bottle (Corning) with 5 ml of IMDM + 10% FBS. On days 1, 3, 5, and 7 of culture, the cells were detached by treatment with pronase-EDTA, and the viable cell number was counted using a hemocytometer. Each value represents the average of counts for 2 bottles.

***Cloning efficiency in soft agar.*** For estimation of cloning efficiency in soft agar, $1 \times 10^3$ cells were suspended in 2 ml of 0.4% LGT agarose (Sigma) in IMDM + 10% FBS and seeded on 2 ml of a 0.6% agar base layer containing IMDM in 35 mm Petri dishes and overlaid with 2 ml of IMDM + 10% FBS. The dishes were then incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. Cells were fed every week with 2 ml of fresh growth medium. Colonies were counted 3 weeks after inoculation under a phase contrast microscope. Cultures were performed in duplicate.

***Assay of tumorigenicity.*** To examine tumorigenicity, $1 \times 10^7$ cells were injected subcutaneously into 6-week-old female athymic nude mice (BALB/cA Jcl-nu) (Clea). All animals were observed for up to 10 weeks,

then they were sacrificed and examined for tumor formation.

***Morphological characterization.*** For histological examination, transplanted tumors, lungs, livers, and mesenteric lymph nodes were fixed with 20% formaldehyde, embedded, sectioned, then stained with hematoxylin and eosin for light microscopy. For transmission electron microscopy, transplanted tumors and cells cultured in 35 mm Petri dishes were fixed in 2.5% glutaraldehyde and processed routinely. Ultra-thin sections were stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (Hitachi H-7100).

***Toxic effect of Fe-NTA.*** Transformed cells and primary cultured renal cells (PRC) were tested. Cells were inoculated at a density of $1 \times 10^5$ cells in collagen-coated 35 mm plastic dishes containing 2 ml IMDM + 10 % FBS. Two days after inoculation, the cells were incubated with fresh medium with or without Fe-NTA (0.5, 2, 5, 10, and 20 $\mu$g of iron/ml). Twenty-four hours later, culture media were collected, then the LDH released in each medium was analyzed with an automatic analyzer (Hitachi Automatic Analyzer 7170). LDH in culture medium treated with 0.3% Triton X-100 (Wako) was estimated as 100%.

Animal experiments were strictly performed in accordance with the Guidelines for Animal Experiments established by Okayama University Medical School.

## Results

***Observation of Transformation.*** In the Fe-NTA-continuously-treated group, when Fe-NTA concentration was less than 2 $\mu$g/ml, almost all of the cells survived, but fibroblastic spindle cells overgrew and replaced the epithelial cells. On the other hand, when cells were treated continuously with more than 5 $\mu$g/ml of Fe-NTA, epithelial cells, which had a cobblestone-like appearance, rapidly disappeared. Almost all of the epithelial cells had died at 2 weeks after the start of treatment. At 4 weeks after inoculation, no transformed cells were observed in this culture schedule. In the Na-NTA treated control group, some cells died in response to the high concentration Na-NTA treatment, but no transformed cells were observed.

In the Fe-NTA-intermittently-treated group, fibroblastic spindle cells overgrew and replaced the epithelial cells with treatment of Fe-NTA at a concentration of less than 2 $\mu$g/ml, which finding was similar to that in the continuously treated group. On the other hand, when cells were treated with more than 5 $\mu$g/ml of Fe-NTA, almost all of the epithelial cells had died at 6 h after the first treatment, though some epithelial colonies appeared among the fibroblastic cells before the second treatment with Fe-NTA. Almost all of these epithelial cell colonies disappeared with subsequent Fe-NTA treatments, though 5 transformed epithelial colonies were recovered in 4 series of experiments at 3 weeks after the first treatment (Fig. 1). The concentrations of Fe-NTA and the treatments that induced transformation were 5 $\mu$g/ml of Fe-NTA 3 times a week, 10 $\mu$g/ml Fe-NTA twice a week, 50 $\mu$g/ml Fe-NTA once a week, 50 $\mu$g/ml Fe-NTA twice a week, and 50 $\mu$g/ml Fe-NTA 3 times a week. No transformed cells were observed in the remaining 6 series of experiments. In the Na-NTA treated control group, some cells died in response to high concentration Na-NTA treatment, but no transformed cells were observed.

Four weeks later, the dishes containing transformed colonies were treated with pronase-EDTA, and detached cells were seeded in plastic bottles. Thereafter, the cells were serially subcultured by pronase-EDTA treatment. Three cell lines were established, and named RK523, RK1124, and RK34. These lines were similar, and all exhibited a polygonal cobblestone-like arrangement with a piled-up focus (Fig. 2).

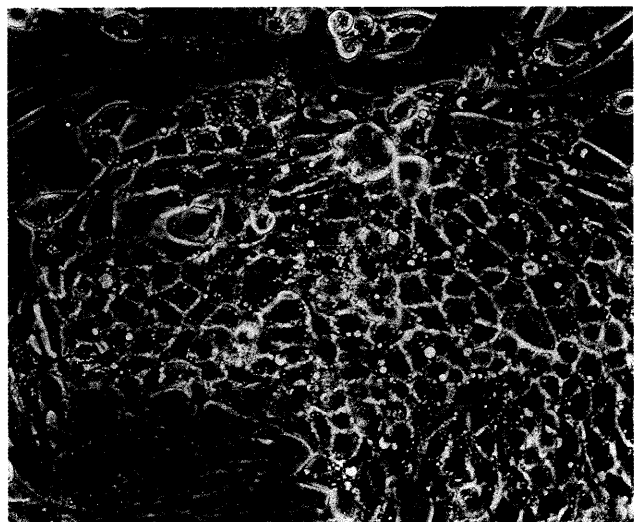

**Fig. 1** Phase contrast micrograph of transformed epithelial colony, observed at 3 weeks after the first treatment.

Electron-microscopically, RK523 cells piled up to form multiple cell layers and had prominent nucleoli, several mitochondria, a rough endoplasmic reticulum, and microvilli on their surfaces and basement membranes (Fig. 3). The RK523 cells grew rapidly compared to the PRC (Fig. 4). The average population-doubling times of RK523 cells and PRC were calculated to be 26 h and 36 h, respectively. The release of LDH from RK523 cells into the culture medium was compared with that of PRC cells by treatment with Fe-NTA (Fig. 5). When Fe-NTA was added to the culture medium of PRC, LDH release was not observed up to 0.5 $\mu$g of iron/ml. LDH release was observed when more than 2 $\mu$g of iron/ml was added to the medium, and more than 60% of LDH was released when 10-20 $\mu$g of iron/ml was added to the medium. On the other hand, when Fe-NTA was added to the culture medium of RK523, only 6.3% of LDH was released at 20 $\mu$g of iron/ml. These results indicate that the RK523 cells could tolerate treatment with a high concentration of Fe-NTA.

Next, we examined whether the RK523 cells acquired malignant characteristics. The RK523 cells grew and formed colonies in soft agar. At 21 days after inoculation of the cells into soft agar, 4-5 colonies/1,000 cells had formed. The diameters of the colonies were 100-700 $\mu$m. Tumorigenicity of the RK523 cells was then tested using athymic nude mice. At 10 weeks after inoculation of cells into athymic nude mice, RK523 cells had formed tumors of about 0.5-1 cm in diameter at a take rate of 5/5, and had metastasized in the lungs of 2 nude mice. RK1124 and RK34 also formed tumors in nude mice, though no lung metastasis was observed.

The histological appearance of the transplanted tumor of RK523 was poorly differentiated carcinoma with high nuclear pleomorphism and no prominent tubular structures (Fig. 6). Electron-microscopically, several tubule-like cell clusters were observed, as shown in Fig. 7, though microvilli and basement membranes were not prominent.

## Discussion

In the present study, we succeeded in transforming PRC by intermittent *in vitro* treatment with Fe-NTA but not by continuous treatment. This may be explained by the fact that PRC were more labile for Fe-NTA than other cell lines were [21, 22]. Thus, even if PRC had acquired tumorigenic mutations by the treatment with Fe-NTA, they would have died in response its to the toxic effects in the continuously treated group. However, when PRC were treated intermittently, they were injured only during the treatment period. Almost all PRC died, though the remaining cells survived and resulted in mutations, and could proliferate until the next Fe-NTA treatment. Some of these cells acquired resistance to

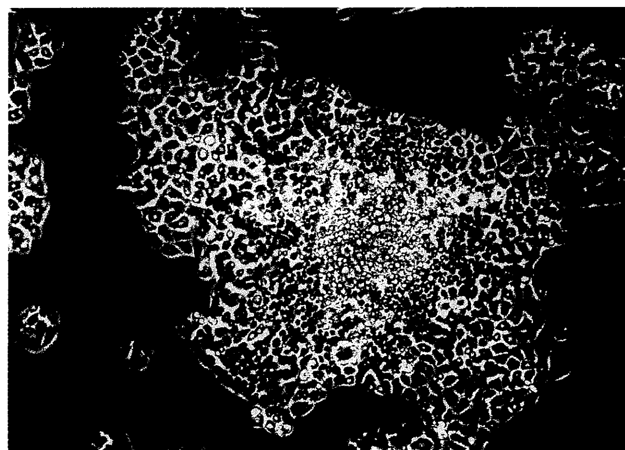

**Fig. 2** Phase contrast micrograph of the RK523 cell line. The RK523 cells have a polygonal cobblestone-like arrangement with a piled-up focus in the central part of the colony growth.

**Fig. 3** Electron micrograph of RK523 cells. Cells were cut perpendicular to the surface of the culture dish. RK523 cells piled up to form multiple cell layers and had microvilli (arrow heads) and basement membranes (arrows). Bar = 5 $\mu$m.

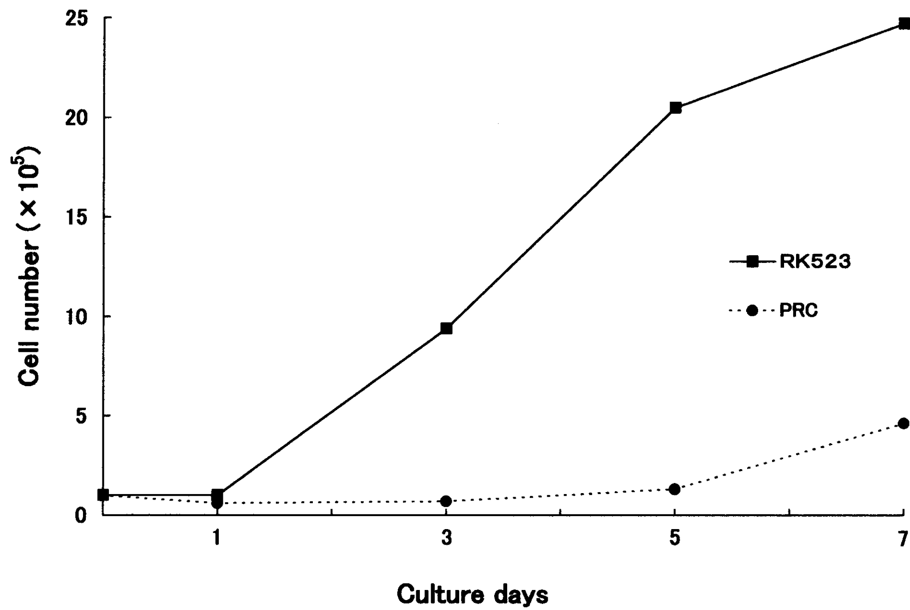


**Fig. 4** Growth curves of RK523 cells and PRC. Each value represents the average for 2 bottles.

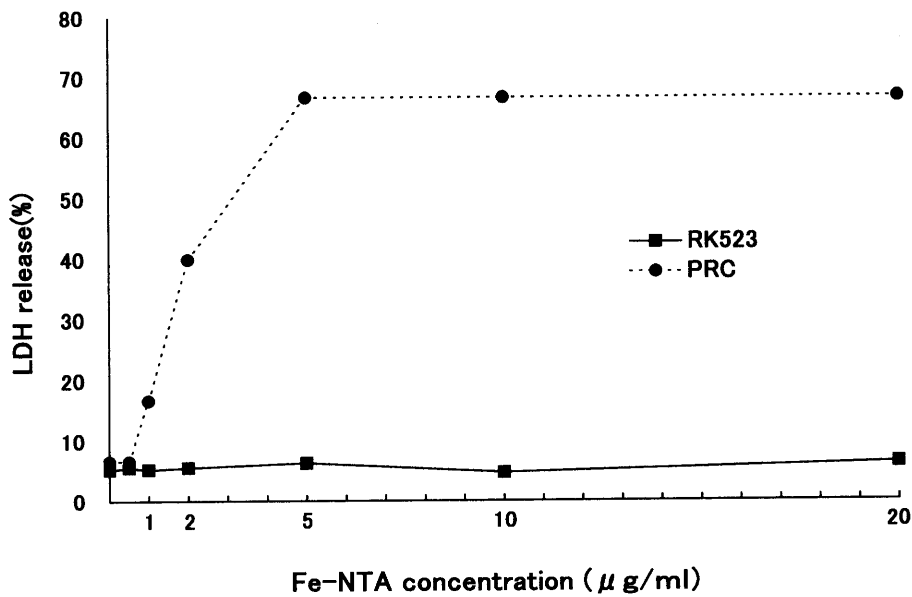


**Fig. 5** LDH release from culture cells by treatment with Fe-NTA. LDH activity in culture medium treated with 0.3% Triton X-100 was estimated as 100%.

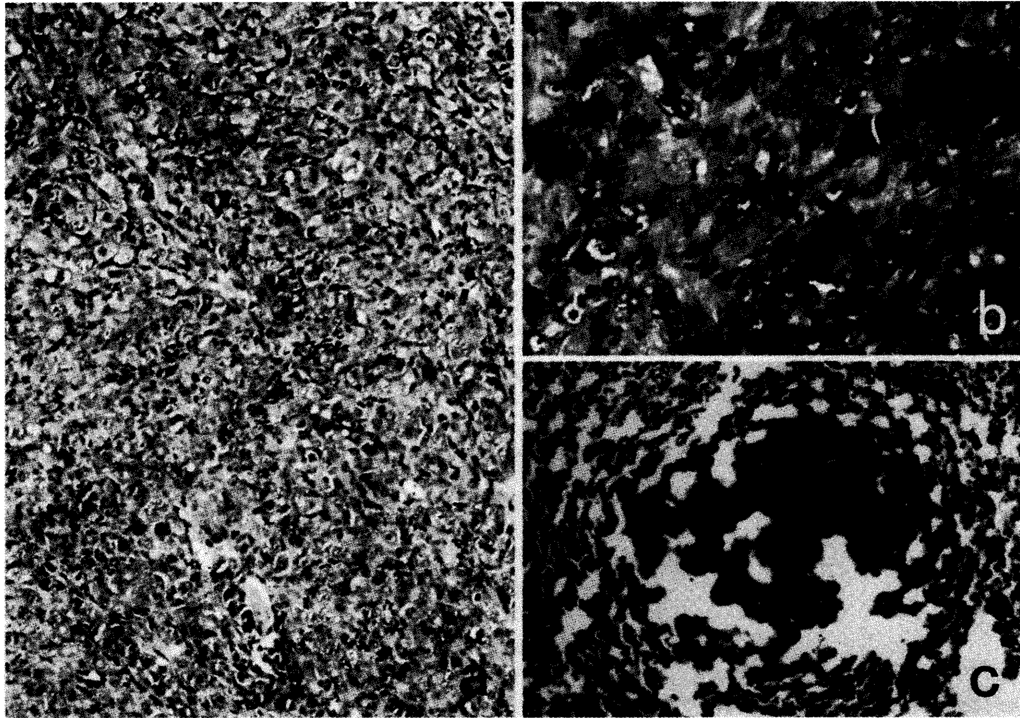


**Fig. 6** Histological appearance of transplanted tumor. a, low magnification, poorly differentiated carcinoma without obvious tubular structures; b, high magnification, pleomorphic tumor cells with atypical mitoses; c, metastatic tumor of the lung.

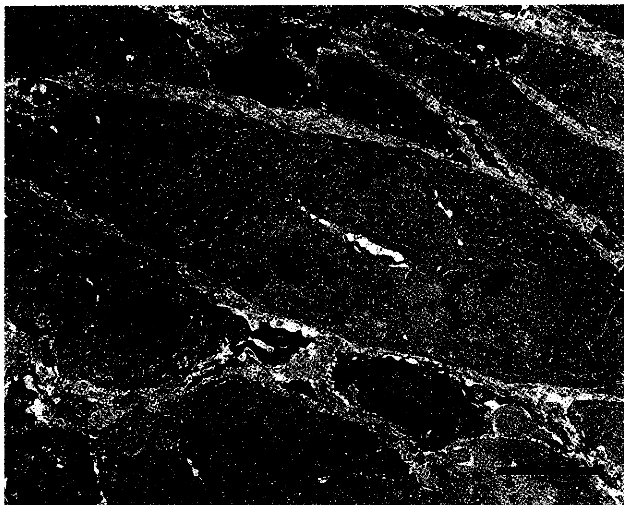

**Fig. 7** Electron micrograph of transplanted tumor. Several tubule-like cell clusters were observed, though microvilli and basement membranes did not develop. Bar = 5 $\mu$m.

Fe-NTA treatment. Finally, repeated treatment with Fe-NTA induced malignant transformation of the cells. These results suggested that Fe-NTA is not only toxic as Kawabata *et al.* [23] reported in *in vivo* administration, but also *in vitro* for renal tubular cells. Notably, the transformation ability of Fe-NTA was not so high as that of other carcinogens such as N-methyl-N'-notro-N-nitrosoguanidine (MNNG) [24]. Therefore, we consider that a balance between cytotoxicity and mutagenecity is important for Fe-NTA-induced transformation.

The characteristics of the RK523 cells include epithelial features with junctions, microvilli, and basement membrane, and malignant characteristics such as piled-up growth, nuclear pleomorphism, tumorigenecity, and metastatic capacity. The histological features of transplanted RK523 cells were poorly differentiated adenocarcinoma. The features of this tumor were similar to those of a cell line established from a renal tumor induced by

repeated injection of Fe-NTA (data not shown). Okada *et al.* reported a similar poorly differentiated component in rat renal tumors induced by Fe-NTA administration *in vivo* [25]. The histological appearance of a transplanted tumor comprised of *in vitro* transformed renal cells has rarely been reported. Walker and Ginsler reported tubular adenocarcinoma established from PRC with MNNG treatment *in vitro* [24]. The difference in histological appearance might be due to the difference in carcinogens.

This *in vitro* transformation system is a useful tool for analysis of the carcinogenic process of Fe-NTA-induced renal tumor, and for examining the effects of reagents such as sex hormones and antioxidants on Fe-NTA-induced transformation of renal cells.

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