Histopathological and Biochemical Analyses of Transplantable Renal Adenocarcinoma in Rats Induced by N-Ethyl-N-hydroxyethylnitrosamine

Yoshio Hiasa, Jung-Chung Lin, Noboru Konishi, Yoshiteru Kitahori, Noboru Enoki, and Taketo Shimoyama

ABSTRACT

Transplantable renal adenocarcinoma can be readily induced in Wistar strain male rats by initiation with N-ethyl-N-hydroxyethylnitrosamine followed by promotion with β-cyclodextrin. The transplantability rates of the tumors by s.c. inoculation in newborn rats were 33 and 50%, respectively, for tumors of the first and second passages, and 100% for both third and fourth passages. The transplantability rates were affected by route of inoculation; rates of 50 and 100% were observed for s.c. and i.p. inoculations, respectively. The growth rate of tumors induced by i.p. inoculation was 3-fold higher than that induced by s.c. injection. Macroscopically, most of the tumors grew in the subcutaneous tissue of inoculation sites. However, invasive growth of tumors in spleen, liver, stomach, peritoneum, and intestine were seen in 50% of the animals inoculated i.p.; metastatic cancers to lung were seen in 16%. Histologically, the tumors were well-differentiated adenocarcinomas composed of uniform cells resembling kidney tubular cells and appeared to be derived from normal kidney tissues. A 5-fold decrease in γ-glutamyl transferase activity in tumor tissues was found as compared with that of nontumorous kidney tissues. Electrophoretic analysis of cellular proteins in polyacrylamide gels revealed that tumor tissues exhibited five new polypeptides with molecular weights of 81,000, 64,000, 59,000, 50,000, and 36,000 which were either lacking or undetectable in the nontumorous area and control kidney. In addition, protein banding patterns of transplantable renal tumor appeared to be more heterogeneous than those of primary kidney tumor.

INTRODUCTION

There are many reports dealing with morphological studies on histogenesis of chemically induced renal adenocarcinomas (1-3, 5, 6, 9, 11, 16, 23, 31). However, morphologically, it is difficult to differentiate renal tubular cell adenomas and renal adenocarcinomas in rats. In human renal adenocarcinoma without remarkable atypia, there is sometimes distant hematogenous metastasis (13). In addition, human renal cell carcinoma (8, 15, 17) could be grown and maintained in nude mice by transplantation. Several reports (14, 24, 25, 28, 29) indicated that rat renal tumors were transplantable. However, transplantable rat renal cell carcinoma with distant metastasis has not been described before. We have confirmed and extended our previous findings that renal tubular cell tumors can be induced in rats by feeding them EHEN2 followed by a s.c. injection of β-cyclodextrin (12). The establishment of transplantable renal adenocarcinoma with the capability of metastasis to other organs and the histopathological and biochemical analyses of this tumor are the subject of this paper.

MATERIALS AND METHODS

Induction of Primary Tumors

The regimen of induction of primary tumor (renal adenocarcinoma) was described previously (12). Briefly, inbred male Wistar rats were fed on a basal diet containing 1000 ppm of EHEN for 2 weeks and followed by a s.c. injection of β-cyclodextrin at a dose of 45 mg/100 g body weight once a day for 7 days. The animals were kept on commercial diet (Oriental MF) beginning from Week 3 to Week 32. Small pale tumors (0.7 x 0.5 cm) developed at the anterior cortex of kidney during this experimental period.

Methods of Transplantation

Renal tumors were removed, minced with a pair of small scissors under sterile conditions, and mashed in 0.9% NaCl (saline) solution. Twelve newborn rats of inbred Wistar strain were given s.c. implants with the same quantity of tumor tissues under cold anesthesia. All animals were weighed and observed daily for tumor development at inoculated sites. Tumor weight was determined at the time of sacrifice. The tumors thus developed in the animals are designated as the first passage. Tumors of the second, third, and fourth passages were obtained by serial transplantations in newborn rats. To determine the effect of route of inoculation on the transplantability rate, young Wistar rats (4-week-old) were given either i.p. or s.c. implants of tumors from the third passage.

Biological Observations

Rats bearing tumors of the first and second passages were killed after 150 days of inoculation. Tumors of the third and fourth passages were removed from the rats as soon as one of the rats died of tumor. The tumors removed were weighed. The tumor tissues were used for transplantation and light- and electron-microscopic studies. Dead and dying animals with tumors of the third and fourth passages were autopsied. The transplantability rate of each passage of tumor was determined as percentage of tumor incidence. Tumor growth rate was determined by tumor weight per duration of inoculation.

Morphological Observations

For light-microscopic studies, tumor tissues were fixed in 10% neutral formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin. Specific stains such as periodic acid-Schiff, Van Giesen, and azan Mallory were also used in some cases.

1 This work was supported by Grants in Aid for Cancer Research from the Ministry of Health and Welfare in Japan (56 sh-2, 58-39) and from the Incentive Wage System in Nara Medical University, and by Institutional Grant IN-15W from the American Cancer Society.
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Received August 11, 1983; accepted December 7, 1983.

[CANCER RESEARCH 44, 1664–1670, April 1984]
Biochemical Studies

**Assay for GGT.** Tissues were homogenized in extraction buffer (0.05 m Tris-HCl, pH 7.4; 0.15 m NaCl; 0.5% Triton X-100; 0.5% deoxycholate; and 1 mM phenylmethylsulfonyl fluoride) followed by sonication and centrifugation at 10,000 rpm for 10 min in a HB-4 rotor in Sorvall. Supernatants were used for enzyme assay. GGT activity was assayed using a Gilford Model 250 recording spectrophotometer. One unit of enzyme releases 1 μmol of p-nitroaniline/min at 37°C from γ-glutamyl-p-nitroanilide and is calculated with the molar extinction coefficient for p-nitroaniline as 10,820 × 10⁶. Protein was determined by the method of Lowry et al. (22).

**Analysis of Cellular Proteins.** To assess whether the development of renal tumor induced by EHEN is associated with the appearance of tumor-specific antigens, we decided, as a first step, to compare the profiles of cellular proteins of tumor and nontumor tissues. Cellular proteins extracted as described above were analyzed on a 7.5% polyacrylamide gel of the system of Laemmli (18). Marker proteins used were γ-glutamyl-p-nitroanilide; and 10 mM glycylglycine to a final concentration of 4 mM L-γ-glutamyl-p-nitroanilide; and 10 mM glycylglycine as acceptor, according to the method of Tate and Meister (30) with slight modification (21).

The reaction mixture contained 50 mM Tris-HCl, pH 8.2; 10 mM MgCl₂; 4 mM L-γ-glutamyl-p-nitroanilide; and 10 mM glycylglycine to a final volume of 2 ml. The change in absorbance at 450 nm was recorded using a Gilford Model 250 recording spectrophotometer. One unit of enzyme releases 1 μmol of p-nitroaniline/min at 37°C from γ-glutamyl-p-nitroanilide and is calculated with the molar extinction coefficient for p-nitroaniline as 10,820 × 10⁶. Protein was determined by the method of Lowry et al. (22).

**Results were average of 2 determinations.**

### Table 1

**Transplantable renal tubular cell tumor in Wistar rats**

<table>
<thead>
<tr>
<th>Passage</th>
<th>Age</th>
<th>No. of rats</th>
<th>WT (g)</th>
<th>Body wt (% of control)</th>
<th>Duration of inoculation (days)</th>
<th>Transplantability rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>1</td>
<td>12 (s.c.)</td>
<td>4</td>
<td>175 ± 61*</td>
<td>33</td>
<td>15.3 ± 9.4</td>
</tr>
<tr>
<td>NB</td>
<td>2</td>
<td>10 (s.c.)</td>
<td>5</td>
<td>185 ± 47</td>
<td>51</td>
<td>54.2 ± 27.8</td>
</tr>
<tr>
<td>NB</td>
<td>3</td>
<td>12 (s.c.)</td>
<td>12</td>
<td>73 ± 21</td>
<td>100</td>
<td>25.0 ± 14.1</td>
</tr>
<tr>
<td>NB</td>
<td>4</td>
<td>11 (s.c.)</td>
<td>11</td>
<td>125 ± 36</td>
<td>100</td>
<td>59.0 ± 25.3</td>
</tr>
<tr>
<td>4w</td>
<td>4</td>
<td>12 (s.c.)</td>
<td>12 (11)</td>
<td>52 ± 18</td>
<td>100</td>
<td>18.5 ± 5.8</td>
</tr>
<tr>
<td>4w</td>
<td>4</td>
<td>8 (s.c.)</td>
<td>4</td>
<td>78 ± 2</td>
<td>50</td>
<td>8.3 ± 2.4</td>
</tr>
</tbody>
</table>

### Table 2

**GGT activity in various kidney tumor and nontumorous tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GGT activity* (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney tumor</td>
<td>0.52</td>
</tr>
<tr>
<td>Nontumor area</td>
<td>2.28</td>
</tr>
<tr>
<td>Transplantable renal tumor</td>
<td>0.57</td>
</tr>
<tr>
<td>Kidney of transplantable renal tumor</td>
<td>2.26</td>
</tr>
<tr>
<td>Control kidney</td>
<td>2.68</td>
</tr>
</tbody>
</table>

* Results were average of 2 determinations.

**Results.** Duration of inoculation, transplantability rates, weights of tumors, and tumor growth rates are summarized in Table 2. The transplantability rates in newborn rats by s.c. inoculation were 33 and 50%, respectively, for tumors of the first and second passages, and 100% for both the third and fourth passages. The growth rates of tumors by s.c. inoculation in newborn rats were 0.36 g/day in the first, 0.34 g in the second, and 0.47 g in the fourth passage.

By comparing the effect of different routes of inoculation, it was found that the transplantability rate by i.p. and s.c. injections were 100 and 50%, respectively, when young rats (4 weeks old) were inoculated with the tumor cells of the fourth passage. The average weight of tumors from transplantable young rats was 18.5 g by i.p. inoculation in contrast to 8.3 g by s.c. inoculation (Table 1). The growth rates of tumors in young rats were 0.36 and 0.1 g/day, respectively, for i.p. and s.c. inoculations.

**Macroscopic Observation.** Macroscopically, most of tumors grew in the s.c. tissue of inoculation sites (Fig. 1). One rat with tumors induced by inoculation of tumor tissues from the fourth passage had invasive growth of tumors into peritoneal areas. Tumor formation in liver, pancreas, and intestinal serosa and adhesion of intestine to liver were observed in all rats inoculated i.p. with tumor tissues from the fourth passage (Fig. 2).

**Light Microscopic Observation.** The primary tumors induced by EHEN and β-cyclodextrin were typical renal adenocarcinoma (Fig. 3). By light microscopy, lymphocytes infiltrations were seen around the transplanted tumors of the first and second passages (Figs. 4 and 5), but not so much around the transplanted tumors of the third and fourth passages (Fig. 6). Cells of transplantable tumors had abundant granular cytoplasm and large round nuclei with clear nucleoplasm similar to cells of the primary tumors. Tumors were surrounded by connective tissues. The transplantable tumor cells formed alveolar or papillary patterns, but not a tubular pattern as found in the primary tumors. Invasion of tumors into spleen (Fig. 7), liver (Fig. 8), intestine (Fig. 9), and stomach were seen in 6 of the 12 rats inoculated i.p. with the tumors of the third passage; metastatic cancers to lung were seen only in 2 of them (Fig. 10).

**GGT Activity.** Table 2 shows that kidney from a control rat had high GGT activity in the crude cell extract with a specific activity of 2.68 unit/mg of protein. The activity of GGT in the nontumorous area and kidney from the rat with transplantable tumor was slightly decreased as compared to that of control kidney. In contrast, a 5-fold decrease in GGT activity was con-

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**References:**

sinctly observed in kidney tumor and transplantable renal tumor.

Electrophoretic Analysis of Cellular Proteins in Polyacrylamide Gels. The cellular proteins isolated from various tumorous and nontumorous tissues were resolved into many components by electrophoresis in the sodium dodecyl sulfate-polyacrylamide slab gels. Fig. 11 shows the results of stained gels which demonstrate several striking differences in the composition of cellular proteins among tumorous and nontumorous tissues. The most conspicuous differences were the presence in primary kidney tumor (gel A) of 5 polypeptides with molecular weights of 81,000, 64,000, 59,000, 50,000, and 36,000 (arrows) which were either lacking or undetectable in the nontumorous area (gel B), kidney from a rat bearing transplantable renal tumor (gel D), and kidney of a control rat (gel E). Interestingly, polypeptides with molecular weights of 81,000 and 59,000 appeared as a doublet in transplantable renal tumor (gel C). In addition, several polypeptides in the higher-molecular-weight region (above 94,000) and 2 polypeptides (M, 28,000 and 20,000) in the lower-molecular-weight region were detectable only in the transplantable renal tumors (gel C).

These differences in protein banding pattern mentioned above could be due to artifacts resulting from overloading the proteins in each slot. To evaluate this possibility, a slab gel loaded with one-half of the amount of the proteins (50 μg/slot) was run under the same conditions. An identical pattern (Fig. 11, I) as the gel loaded with 100 μg/slot (Fig. 11, I) were observed.

DISCUSSION

In the present study and in our previous work (10-12), we have demonstrated that the transplantable renal adenocarcinoma can be readily induced in male Wistar rats initiated with EHEN followed by promotion with β-cycloexetrin. There are several reports describing the induction of transplantable renal adenocarcinoma (6, 24, 25) and nephroblastoma (6, 14, 28, 29). The transplantable renal adenocarcinomas reported by Morris et al. (24) possessed different rates of growth, and these tumors were histologically of a low order of cancer which did not metastasize into other organs. In contrast, the transplantable renal adenocarcinoma induced under our experimental regimens were highly malignant tumors which metastasized to lung and invaded to several organs such as spleen, liver, intestine, and stomach. However, the primary tumors induced by EHEN alone (11) or either EHEN and β-cycloexetrin (12) or EHEN and basic lead acetate (10) were unable to metastasize. Tubular formation and papillary proliferation of tumor cells were often seen in the primary tumor but only occasionally seen in the transplantable tumor. EHEN induced 2 types of renal tubular cell tumors, the clear and dark cell type. Cells of the transplantable tumors that formed sheet-like structures were dark cells; they exhibited morphological similarity to that of the dark cells of the primary tumor.

The transplantability rates of tumors of the first and second passages were, respectively, 33 and 50% but increased to 100% in the third and fourth passages. The growth rates of the transplantable tumors were low in the first passage but high in the second, third, and fourth passages. These results, taken together, indicate that the tumor cells may become more malignant resulting from serial transplantations in vivo. Depending on the route of inoculation, tumor cells of the fourth passage showed invasive growth into peritoneum when inoculated s.c. and into liver, pancreas, and lung when inoculated i.p.

The most important findings in this study are the correlation of the histopathological alterations with some biochemical changes. GGT is an enzyme widely distributed in rat tissues, mostly in the kidney with only one-twentieth as much as in the liver and less in other tissues. The activity in fetal kidney is approximately one-fifth of the adult's.4 Our previous report (26) indicated a marked decrease in GGT activity in rat renal cell neoplasms as determined by histocytotoxic staining. The present observation provides quantitative data indicating that GGT activity was decreased approximately 5-fold in primary kidney tumor and transplantable renal tumor. The functional biochemical significance of this alteration in GGT activity in kidney tumor tissues is unknown. However, these results may suggest the presence of abnormal gene expression, probably activated during the process of carcinogenesis.

Analysis of cellular protein constituents on polyacrylamide gels clearly shows that 5 new polypeptides (M, 81,000, 64,000, 59,000, 50,000, and 36,000) appeared in primary kidney tumors. Interestingly, additional changes in protein banding patterns, such as formation of doublets of polypeptides with molecular weights of 81,000 and 59,000 and appearance of several new polypeptides in the higher- and lower-molecular-weight regions, were observed only in the transplantable renal tumors. Increasing the complexity of the protein banding patterns seems to correlate with the phenotypical changes characteristic of malignant neoplasia, such as autonomous growth, invasion, or metastasis, or even atypical cytological aberrations.

Neoplastic growth was shown to change the relative proportions of several enzymes, isoenzymes, and antigenic components (4, 7, 19, 20, 27). In general, such changes resemble the patterns seen in fetal tissues. It is tempting to consider that these and perhaps other associated changes may appear as a "functional package," conceivably due to some permanent change in one or more regulatory genes or gene clusters, and may have some role to play in the progressive evolution to malignant neoplasm.

ACKNOWLEDGMENTS

We thank Dr. Joseph S. Pagano for reading the manuscript and Barbara Leonard for typing.

REFERENCES

Analyses of Renal Adenocarcinoma


Fig. 1. Macroscopic picture of s.c. tumors of the third passage.
Fig. 2. Macroscopic picture of peritoneal tumors of the fourth passage.
Fig. 3. Histological picture of the primary tumors. H & E, x 100.
Fig. 4. Histological picture of s.c. tumors of the first passage. H & E, x 100.
Fig. 5. High magnification of Fig. 4, showing the proliferation of lymphocytes around the tumor cells. H & E, x 200.
Fig. 6. Histological picture of s.c. tumor of the fourth passage. H & E, x 100.
Fig. 7. Histological picture of tumor of the fourth passage in spleen obtained by i.p. inoculation. H & E, × 100.
Fig. 8. Histological picture of tumor of the fourth passage in liver obtained by i.p. inoculation. H & E, × 100.
Fig. 9. Histological picture of tumor of the fourth passage in small intestine obtained by i.p. inoculation. H & E, × 100.
Fig. 10. Histological picture of metastatic tumor to lung. H & E, × 100.
Fig. 11. Electrophoretic analysis of cellular proteins on polyacrylamide gel. Proteins were extracted from various tumor and nontumor tissues as described in the text. After electrophoretic separation, the protein bands were revealed by staining with Coomassie blue. I, 100 μg protein/slot were applied; II, 50 μg protein/slot were applied. A, primary kidney tumor; B, nontumorous area; C, transplantable renal tumor; D, kidney from rat bearing transplantable renal tumor; E, kidney of control rat; S, standard marker proteins. M.W., M.
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