Experimental Model for Combination Chemotherapy with Metronidazole Using Human Uterine Cervical Carcinomas Transplanted into Nude Mice

Hisashi Tokita, Noboru Tanaka, Kazuyoshi Sekimoto, Tetsuo Ueno, Karoku Okamoto, and Shinji Fujimura

Division of Animal Research [H. T.], Department of Pathology [N. T., K. S., T. U.], Division of Chemotherapy [K. O.], and Division of Biochemistry [S. F.], Chiba Cancer Center Research Institute, 666-2, Niltona-cho, Chiba 280, Japan

ABSTRACT

Human uterine cervical carcinomas (Yumoto strain) and HeLa cell tumors were transplanted into nude mice, and their transplantable strains were established. The fundamental histological features of these tumors were analyzed according to their histological construction and cytological maturation. The effect of administered drugs was examined morphologically. The Yumoto strain is a well-differentiated epidermoid-type carcinoma consisting of regularly arranged basal-type, parabasal-type, and keratinizing-type cells. The HeLa cell tumor is made up of solid medullary carcinoma cell nests in which trabecular arrangements begin to appear around the medullary areas after the third passage. This feature is maintained up to the 17th generation. The basal layer-type cancer cells of the Yumoto strain as well as trabecularly arranged cancer cells in the HeLa cell tumor were selectively influenced by administration of bleomycin and/or mitomycin and showed considerable degeneration or complete disappearance. On the contrary, metronidazole (a drug for vaginal trichomoniasis; Flagyl) displayed a cytotoxic effect on the parabasal-type and/or more mature cancer cells of the Yumoto tumor as well as on the solid medullary area of the HeLa cell tumor. This result may indicate a selective affinity of drugs for malignant cells according to their histological construction, and it is conceivable that these types of carcinoma can be affected by combination administration of metronidazole and oncostatic chemicals such as bleomycin and mitomycin. This speculation was realized in this experimental animal research.

INTRODUCTION

After the introduction of a congenitally athymic hairless mutant mouse, the "nude mouse," successful transplantation of various kinds of human tumors as well as cultured cells has become possible, and heterologous transplantable strains have been established (3, 7, 10). Since 1974, we have been investigating biological characteristics, morphological changes, and sensitivity to various kinds of antitumor agents (including irradiation) in human tumors transplanted in nude mice, such as uterine cervical carcinomas of a well-differentiated keratotic epidermoid type (Yumoto strain), HeLa cell tumors, malignant melanomas, and other bone and soft-tissue tumors (1, 13). The human tumors transplanted in nude mice have maintained their histological resemblance to the original primary tumors (7, 10). It is believed that these transplanted human tumors present good models for experimental oncostatic therapeutic research. In this report, the analysis of the histological patterns of Yumoto strain and HeLa cell tumors are described. In addition, the different responses to various kinds of antitumor agents were analyzed morphologically according to histological construction.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female BALB/c nude mice were provided for this study. These specific-pathogen-free nude mice were obtained from The Central Institute for Experimental Animals, Kawasaki, Japan, and maintained by the barrier system. Three to 6 mice were used for each experimental group.

Original Human Tumor for Transplantation and Method of Implantation. Small tissue blocks of uterine cervical carcinoma were obtained from the surgical material of a 46-year-old female (Yumoto) within 1 hr after hysterectomy. This was a well-differentiated epidermoid carcinoma of the keratinizing type. The specimens were subsequently rinsed with 70% ethyl alcohol, 0.5% sodium hypochlorite and phosphate-buffered saline (pH 7.2). Then they were sectioned into small pieces of approximately 2 x 2 mm for implantation. These small tissue blocks were implanted into the s.c. tissue of 3 nude mice at the dorsum by using a trocar.

HeLa Cells. The cultured cell line of HeLa cells was obtained from the National Institute of Health, Tokyo, Japan. This cell line was maintained in Eagle’s minimum essential medium. The cells 1 x 10⁷ were suspended in phosphate-buffered saline and then injected into the s.c. tissue of the nude mice at the dorsum.

Consecutive Transplantation. A portion of the tumor nodules that developed in the mice was excised under sterile conditions. This specimen was immediately subdivided into small fragments and injected into other nude mice.

Drugs Used. BLM3 and MMC were obtained commercially. BLM was injected i.p. at 10 mg/kg body weight once a week, and MMC was injected i.p. at 2 mg/kg body weight once a week. ME (a therapeutic drug for vaginal trichomoniasis; Flagyl) was supplied by Shionogi Pharmaceutical Co., Oosaka, Japan. ME was given p.o. at the rate of 10 mg/kg 2 to 3 times a week.

Administration of these drugs was started approximately 2 weeks after tumor inoculation when the implanted tumor node was between 5 and 15 mm in diameter. Drugs were given continuously for 2 to 8 weeks according to the schedule shown in Charts 2, 3, and 4.

Combination Therapy. For evaluation of the effect of combination administration of these 3 kinds of drugs, the schedule was as follows: BLM plus ME for the Yumoto strain and the HeLa cell tumor, BLM plus MMC for the Yumoto strain (Chart

1 This work was partly supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Tokyo, Japan.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: BLM, bleomycin; MMC, mitomycin; ME, metronidazole.

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the 44th generation. No spontaneous tumor regression was observed except in cases when the tumor was injured due to scratching by the animals themselves. Around the period when the tumor became as large as 15 mm at its greatest diameter, the mice began to lose weight (Chart 1). They rapidly developed a cachectic condition. This phenomenon appears to present very informative parameters for evaluating the effect of an administered oncostatic agent. At the present time, about 80% of the animals have died within 3 months after transplantation.

**Histological Findings.** Histologically, the mice showed well-differentiated epidermoid carcinomas with features that were fairly similar to the features of the original patient carcinoma. Throughout the sequential transplantations, these histological features maintained their original patterns. Regularly arranged areas of parakeratosis, central cornification, and parabasal-type cancer cells were surrounded by an actively growing basal cell-type cancer cell layer (Figs. 2 and 5). The areas composed of parabasal-type cancer cells appeared to become dominant after the eighth generation. These cells have relatively large cytoplasm and ovoid nuclei with distinct, enlarged nucleoli (Fig. 5). Active tumor proliferation appeared to be displayed mostly in the basal and parabasal zones. Mitotic figures, often abnormal, were frequently observed, particularly in the basal cell layer. The extent of necrosis had a direct relationship to tumor size. Thus, the larger tumors exhibited an extensive and confluent central necrosis containing abundant keratotic debris.

**Effect of Individual Drugs.** Administration of BLM or MMC yielded a slight inhibition of tumor growth (Chart 2). However, none of the cases showed tumor regression. The cachectic condition was improved by MMC. Histologically, BLM was disclosed to have selective affinity for the basal-type cancer cells, which showed considerable change characterized by marked degeneration. However, the parabasal cell area and the more-differentiated intermediate-type cell area were not influenced or damaged (Fig. 6). Many keratinizing cells were present. MMC caused similar degenerative changes, but fewer keratinizing cells were present (Fig. 7).

The administration of ME had no significant influence on tumor growth (Chart 2). However, the characteristic significant degenerative change and necrosis, with selective affinity for the parabasal cell-type and/or more mature cancer cells, was observed histologically (Fig. 8), whereas the basal layer cancer cells remained without regressive change and are still active.

**RESULTS**

**Yumoto Strain**

**The First Generation.** Three weeks after the initial implantation of the specimen taken from the hysterectomized material of a human uterine cervical carcinoma, tumor growth became recognizable at the site of transplantation. The tumor continued to grow slowly (Fig. 1). Histological examination 36 days after implantation revealed features very similar to those of the xenografts of the original patient's uterine cervical carcinoma (Figs. 2 and 3).

**After the Second Generation.** Tumor growth became noticeable 2 weeks after implantation. A more rapid growth rate was seen than in the first generation. Transplantability in nude mice ranged from 80 to 100%. Chart 1 shows the growth curves of the Yumoto strain in nude mice of the fifth generation. The tumors were ovoid and sometimes displayed a cauliflower-like appearance (Fig. 4). In all cases, tumor growth was limited only to the implanted site. Lymph node and remote organ metastasis has not been observed up to the present time, i.e., the 44th generation. No spontaneous tumor regression was observed except in cases when the tumor was injured due to...
Combination Administration. To investigate the possibility suggested by these findings, namely, the selective affinity and sensitivity of drugs for components in the topographical histological construction of cells, combinations of ME and BLM and/or MMC were administered. Simultaneous administration of BLM and ME disclosed that Yumoto carcinoma tissue showed highly advanced necrosis, particularly the near complete depletion of basal and parabasal layers (Fig. 9). The former appears to be affected by BLM, and the latter appears to be affected by ME. Carcinoma cells showed uniform chromatin condensation indicative of the tendency of the degenerative process. Large bizarre cells were frequently encountered. Whereas tumor growth as presented by the growth curve appeared to be inhibited only to a slight degree, histology disclosed considerably advanced tumor necrosis with multicystic and hemorrhagic foci. However, administration of BLM plus MMC caused the tumor size to remain constant (Chart 3). Histologically, the basal layer was almost completely depressed or had disappeared, while parabasal and/or more mature cell components remained without necrosis (Fig. 10). After administration of BLM plus MMC plus ME, the tumors in 75% of the animals had disappeared macroscopically (Chart 4). Even microscopically, no remains and/or surviving tumor cells could be detected.

HeLa Cell Tumor

Macroscopic Appearance. The rate of success in transplantation of the HeLa cell tumor into the nude mice was always 100%. From the first generation, the latent period after inoculation of the cultured cell suspension was relatively short and fairly constant until the 17th generation. Approximatley 1 week after injection, a patchy nodule became palpable at the injection site and continued to grow. Chart 5 shows the growth curves of transplanted HeLa cell tumors into nude mice. The tumors growing in the s.c. tissue were well-circumscribed ovoids with an elastic consistency. The cut surface showed a homogeneous grayish appearance (Fig. 11). The overlying skin appeared generally intact and freely movable against the tumor. The tumor volume usually became as large as 200 cu mm within 2 or 3 weeks after transplantation. The tumors continued their growth, reaching a weight of as much as 30 g up to the sixth month after transplantation, with the tumors often becoming larger than the bodies of the tumor-bearing mice themselves. However, the animals never developed a cachectic condition. They generally lived longer than 6 months even though they were burdened by such huge tumors. In some cases, pulmonary metastasis was detected by autopsy.

Histological Findings. During the first and second generations, histological examination revealed medullary anaplastic neoplastic tissue with an occasional pseudoglandular structure. The neoplastic cells were uniform in size. Mitotic figures were frequent (Fig. 12), and abundant periodic acid-Schiff-positive glycogen granules were detected in the cytoplasm. Alcian blue and mucicarmine staining showed negative findings.

After the third generation, the tumor cells tended to form 2 different arrangements: (a) medullary solid nests in the central areas of the cancer nests; and (b) a partially trabecular arrangement in the peripheral zone of the nests. Foci of various degrees of necrosis were observed, especially in the central portion of the tumors. After the 13th generation, tubular and/or cord-like cell ar-
rangement became more dominant, and Alcian blue- and/or mucicarmine-positive material was detectable in the cytoplasm. Such mucoid material was seen to be confluent, forming a cystic space. This tendency became more significant by the 17th generation (Figs. 13 and 14).

**Effect of Individual Drugs.** Administration of BLM to the animals bearing HeLa cell tumors resulted in disappearance of the areas of trabecular arrangement, but solid medullary tumor nests remained uninfuenced. Histology of this HeLa cell tumor on the seventh day of final administration of BLM is shown in Fig. 15.

On the contrary, administration of ME caused degenerative changes and necrosis, with the trabecular arrangement remaining unchanged (Fig. 16).

**Combination Administration.** Six weeks after the administration of both BLM and ME, inhibition of tumor growth appeared and continued for a long time during administration, but no cases showing complete regression of tumor were recognized. Histologically, trabecular arrangements and solid medullary tumor nests were found to disappear, but a small number of cancer cells steadily remained (Fig. 17). These findings reflected our initial speculation regarding combination therapy, namely, that the combined administration of different drugs would prove to have significant effects.

**DISCUSSION**

The histological patterns of transplantable animal tumors are often variable in consecutive transplantations. However, human tumors transplanted in nude mice generally maintain their histological resemblance to the primary original tumors throughout (7, 10). We recognized this fact because of the minute histologically, trabecular arrangement of solid medullary tumor nests detected by histological examinations in our experimental series (1). Histologically, trabecular arrangements and solid medullary tumor nests were found to disappear, but a small number of cancer cells steadily remained (Fig. 17). These findings reflected our initial speculation regarding combination therapy, namely, that the combined administration of different drugs would prove to have significant effects.

**REFERENCES**

3. Fogh, J., Fogh, J. M., and Orfeo, T. One hundred and twenty seven cultured animal research suggests that ME is an effective drug in cancer chemotherapy of epidermoid carcinomas in combination with oncostatic chemical agents such as BLM or MMC.

1. Anticancer drugs such as BLM and MMC affect the outer zone of the carcinoma nests, the parabasal cell zone and more mature areas in the Yumoto strain, and the solid medullary nests of the HeLa cell tumor were tremendously affected by ME, resulting in the death of these cells. These areas are more distant from the fibrovascular stroma, thereby indicating that they are in a more hypoxic condition. This result appears likely to be synonymous with the observation of Sutherland (12). The difference in the degree of toxicity of this drug in the cells very probably depends on the grade of hypoxia of the tumor cells. This phenomenon appears to be reflected in the killing action of ME on the trichomomas as well as on anaerobic bacteria.

The histologically detected site of response for ME is apparently different from that for chemical anticancer drugs. Whereas anticancer drugs affect the cancer cells composing the outer zone of the cancer nests or those close to the blood vessels, ME has an affinity for hypoxic cells, which are quite removed from the fibrovascular stroma. Thus, this experimental animal research suggests that ME is an effective drug in cancer chemotherapy of epidermoid carcinomas in combination with oncostatic chemical agents such as BLM or MMC.


**BLM, MMC, and ME in Tumors Transplanted in Nude Mice**

Fig. 1. First passage of Yumoto carcinoma 36 days after transplantation.

Fig. 2. Section from human donor material of Yumoto carcinoma. Well-differentiated epidermoid carcinoma. H & E, x 400.

Fig. 3. First passage of Yumoto carcinoma. Note marked resemblance to the primary tumor shown in Fig. 2 H & E, x 400.

Fig. 4. Macroscopic findings of the eighth passage of Yumoto carcinoma 71 days after transplantation. Cauliflower-like appearance.
Fig. 5. Histology of the untreated Yumoto carcinoma (the ninth passage). Thickening of the parabasal cell layer. H & E, x 200.

Fig. 6. Histology of the BLM-treated Yumoto carcinoma. H & E, x 200.

Fig. 7. Histology of the MMC-treated Yumoto carcinoma. H & E, x 200.

Fig. 8. Histological section of ME-treated Yumoto carcinoma. Marked decrease in cell population of central area is obvious. H & E, x 200.
Fig. 9. Histology of the combination-treated Yumoto carcinoma. BLM plus ME. H & E, x 100.

Fig. 10. Histology of the combination-treated Yumoto carcinoma. BLM plus MMC. H & E, x 200.

Fig. 11. Gross appearance of HeLa cell tumor 27 days after injection of $1 \times 10^7$ HeLa cells.

Fig. 12. First passage of HeLa cell tumor. Medullary neoplastic tissue with scant pseudoglandular structure and active mitosis. H & E, x 200.
Fig. 13. Histology of untreated HeLa cell tumor of the 17th generation. Solid tumor nests. H & E, x 200.

Fig. 14. Histology of untreated HeLa cell tumor of the 17th generation. Pronounced trabecular arrangement. H & E, x 200.

Fig. 15. Histology of the BLM-treated HeLa cell tumor. H & E, x 200.

Fig. 16. Histology of the ME-treated HeLa cell tumor. H & E, x 100.

Fig. 17. Histology of the combination-treated HeLa cell tumor. BLM plus ME. H & E, x 400.
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