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A Comparative Study of Cell Proliferative Activity between the Primaty Tumor and the Metastatic Lymph Node

Kosei Miyashita

First Department of Surgery, Nagasaki University School of Medicine 7-1, 1-chome, Sakamoto, Nagasaki, Japan

Summary: Differences in the proliferative activity of cancer cells between the primary lesion and the metastatic lymph node of solid tumors were studied in subcutaneously transplanted rat tumor AH109A and human gastric cancer. The cellular RNA content (RNA index, RI), colony formation assay (plating efficiency, PE) and BrdUrd labeling index (LI) were used as indicators for the proliferative activity of cancer cells. In the experiment with transplanted rat tumor, RI, PE, and LI fell off significantly over time at l, 2 and 3 weeks after transplantation. On comparison of the primary lesion and the metastatic lymph node, the DNA index (DI) remained unchanged but RI increased significantly in the metastatic lymph node. PE and LI likewise showed significantly high values in the metastatic lesion. When the DNA-RNA indices for 19 cases of human gastric cancer were studied, DI remained almost unchanged in the primary lesion and the metastatic lymph node but RI showed high values in the lymph node. Therefore, the proliferative activity of cancer cells is considered to be higher in the metastatic lymph node than in the primary lesion, which is very significant for the progression of the tumor. On the other hand, chemotherapy can be expected to be more effective in the metastatic lymph node than in the primary lesion. The present study may provide a theoretical basis for reduction surgery on the primary lesion followed by other techniques on the secondary lesion.

Introduction

The most noteworthy characteristics of cancer are represented by biological features — invasion and metastasis. Of these, metastasis is a factor that changes cancer from a local disease to a general disease, and the presence of metastasis makes local treatment, including surgery and radiotherapy, difficult, requiring a multidisciplinary treatment including chemotherapy. Metastasis can be roughly divided into the two categories of hematogenous metastasis and lymphogenous metastasis. Lymphatic metastasis, one type of lymphogenous metastasis, is clinically the most frequently encountered and its extent is considered important as a prognostic factor. (1)

Regarding this metastasis and the lymph node, there are many studies conducted from a viewpoint centering around lymphocytes within the immunological approach since the lymph node is an important center of immune response in the living body (2) (3) (4) (5) (6) (7) (8). However, there seem to be few studies on the chracteristics of the metastatic cancer itself. Of the studies conducted with regard to the nature of the of cancer cells, many deal with the characteristics of the primary lesion and few deal with the characteristics of the cancer cell itself in the lymph node, the place of metastasis. In clinical practice, we often encounter cases where the metastatic lesion advances faster than the primary lesion and cases where there is a difference in efficacy between the primary lesion and the metastatic lesion even when the same anti-cancer drug is used. It is of great interest to see whether the cells constituting the metastatic lesion and the primary lesion are identical and what is the proliferative activity of the cancer cells concerned. At present, however, there are few studies that deal with the proliferative activity of cancer cells in the primary lesion and the metastatic lymph node from such a viewpoint. To clarify the differences in the proliferative activity of cells between the primary lesion and the metastatic lymph node, a study was undertaken with subcutaneously transplanted rat tumor as the model of lymphatic metastasis and lymphatic metastasis of human gastric cancer, using the cellular RNA content, colony formation assay and BrdUrd labeling index as the markers for cell proliferation. Differences in DNA ploidy were also studied to see the cell structure of the primary lesion and the lymph node.

Materials and Methods

(1) Experiment with the rat model

1) Experimental animals and the tumor

Male Donryu rats SPF (specific pathogen-free, Nippon Rat) weighing 120 g 5 weeks after birth were used in the experiment. The tumor used was rat ascitic cancer of the liver AH109A supplied by the Department of Oncology, Research Institute for Tuberculosis and Cancer, Tohoku University. The mortality rate for 1.0×10^5 cells of this tumor transplanted abdominally is 100 percent, and the animals die on average 12 days after transplantation. When 1.0×10^6 cells of this tumor are transplanted subcuta-

neously into the dorsal part of the rat paw, the tumor volume increases exponentially up to about 15 days after transplantation and reaches a plateau thereafter; the animals die of this tumor in about 30 days. As regards lymphatic metastasis, metastasis occrus in almost 100 percent of cases with transplantation of 1.0×10^6 cells (9).

2) Methods

a) Transplantation of the tumor

Ascites containing cells in the exponential phase at 5 to 7 days after abdominal transplantation of the tumor were collected with a 1-ml syringe and diluted in RPMI 1640 (GIBCO) with 10% FCS to prepare a cell suspension so that 1×10^6 cells were contained in 0.1 ml. This cell suspension containing 1×10^6 cells was injected subcutanously into the dorsal part of the rat hind paw using a 1-ml injector with 27 gauge for transplantation. The animals were sacrificed and the primary lesion was collected one, two and three weeks after transplantation to see the progress of the tumor and additionally the metastatic lymph node was collected together with the primary lesion at the third week.

b) Sample preparation

The tumor was measured for the major axis (a) and minor axis (b) with callipers and the size was expressed as 1/2 ($a \times b^2$). After that, the sample was halved. One half was used for histology and the lymph node was histologically confirmed to be positive for metastasis. The other half was incubated in RPMI 1640 supplemented with 0.25% trypsin (SIGMA) at room temperature for 10 minutes to prepare a single cell suspension.

c) Measurement of DNA-RNA content

The DNA-RNA content was measured by flow cytometry using acridine orange staining (AO) according to the method of Darzynkiewicz et al. (10) with necessary modifications. Briefly, 1×10^6 cells fixed in ethanol-aceton (1:1 v/v) were added to 0.01% acid detergent on ice. With an acridine orange solution added 30 seconds later, staining was done at room temperature for 5 minutes. FACS IV (Becton Dickinson) was used for measurement, and excitation was effected with an argon laser of 488 nm. Fluorescence at 530 nm was taken as the DNA content and that at 640 nm as the RNA content; 20,000 cells were counted and indicated on a dot plot with the DNA content plotted along the ordinate and the RNA content along the abscissa. A histogram was also prepared. The DNA-RNA indices (DI, RI) (11) (12) were calculated as a relative indicator for the cellular DNA-RNA content. That is, the DNA-RNA indices equals the G_0G_1 peak of cancer cells/G_0G_1 perk of lymphocytes. (Fig. 1)

d) Colony formation assay

Tumor cells at a concentration of 1×10^5 in 10% FCS-added RPMI 1640 supplemented with 0.33% ager (DIFCO) were scattered on a 6-well plate and incubated under 5% CO_2 at 37 ℃ . After 7 to 10 days, colonies with more than

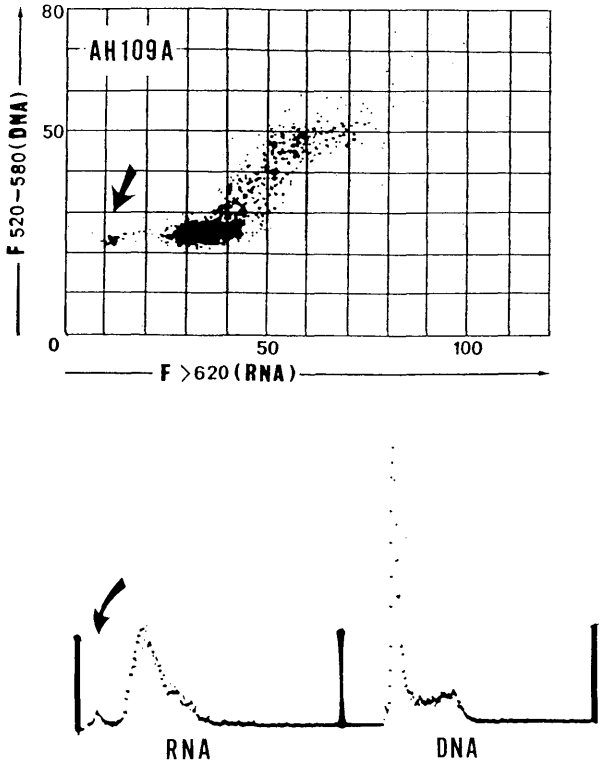

Fig. 1. Dot plot and DNA-RNA histograms of AO stained AH109A cells by flow cytometry. Arrows indicate lymphocytes in dot plot and RNA histogram respectively. AH109A cells can be easily discriminated form lymphocytes in RNA value although DNA values of both cells are approximately the same.

50 cells were counted and plating efficiency (PE) was calculated.

e) BrdUrd Labeling Index

Bromodeoxyuridine (BrdUrd), an analogue of thymidine was administered and its labeling index was calculated to evaluate the quantity of S-phase cells in the cell cycle. That is, BrdUrd (SIGMA), 50 mg/kg was administered intraperitoneally to rats immediately before sacrificing, pulse labeling was done for 1 hour and the primary lesion and the metastatic lymph node were collected. These were fixed with 70% ethanol and embedded in paraffin to prepare 4 μm sections. Subsequently, immunohistochemical staining was performed by the ABC method (VECTOR) using anti-BrdUrd monoclonal antibody (Becton Dickson) (13). Ten visual fields were selected randomly, labeled cells per 1000 cancer cells were counted, the percentage thereof was found and the labeling index (LI) was calculated. (Fig. 2)

(2) Experiment with human gastric cancer

About 1 g of the margin of the primary lesion and macroscopically definite metastatic lymph node were collected from the surgical specimen. These samples were minced into 1 mm sections with a scalpel in RPMI 1640 with 10% FCS. The sections were stirred in an enzyme mixture of

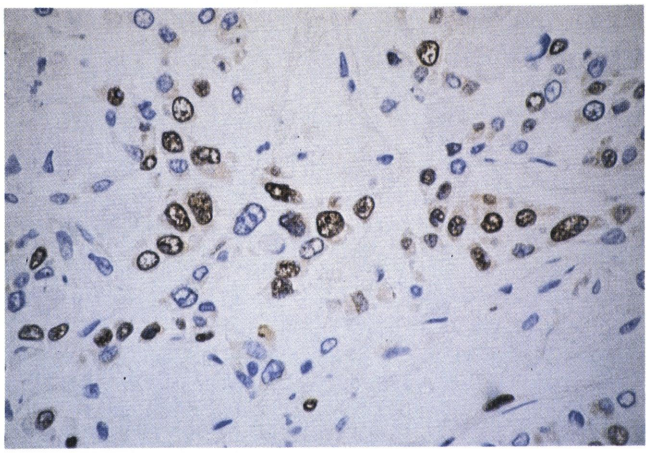

Fig. 2. Tissue section reacted with anti-BrdUrd monoclonal antibody followed by staining with ABC method. The section was counterstained with hematoxylin. BrdUrd incorporated cells have brown nuclei. Magnification x400.

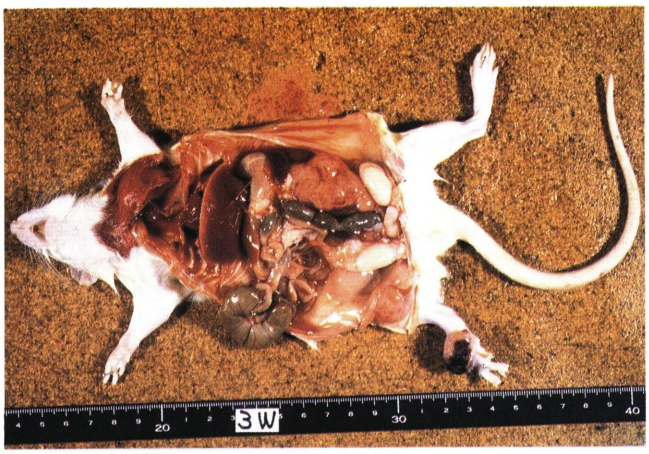

Fig. 3. Photomicrograph of lymph node metastasis of AH109A transplanted Donryu rat. Marked inguinal and abdominal metastatic lymph nodes are visible.

0.02% Collagenase IA (SIGMA) and Dispase 3000 U/ml (SIGMA) at 37 ℃ for 2 hours and filtered through a 40 μm nylon mesh to make a single cell suspension. In case the proportion of normal cells such as lymphocytes was high, the ratio of tumor cells was raised by the Ficoll Conray discontinuous density gradient method (PHARMACIA) (14). After wasihng with PBS, the cells were fixed with ethanol-acetone (1:1 v/v), stained with acridine orange, the DNA-RNA content was measured and the DNA-RNA indices were calculated in the same way as for rats.

(3) Statistical treatment

The Chi-square test was used for testing significant differences over time between the groups and the Wilcoxon test for testing significant differences between the primary lesion and the metastatic lymph node. Correlation coefficients were determined by linear regression analysis.

Results

(1) Experiment with the rat model

With subcutaneous transplantation of 1 × 10^6 cells of AH109A into the dorsal part of the paw, an evident lymphatic metastasis was noted in the popliteal lymph node, inguinal lymph node and abdominal lymph node after three weeks (Fig. 3). Metastasis was also found in the axillar lymph node in cases where metastasis was remarkable.

a) Length of time after transplantation and cell proliferative activity

The relationship between the length of time after transplantation and three indicators (the DNA-RNA indices, colony formation assay and BrdUrd labeling index) was studied as to the primary lesion. The DNA index showed no change during each period after transplantation, while RI fell off with time after transplantation and showed significantly low values three weeks after transplantation (Fig. 4a). When the plating efficiency, an indicator for colony forma-

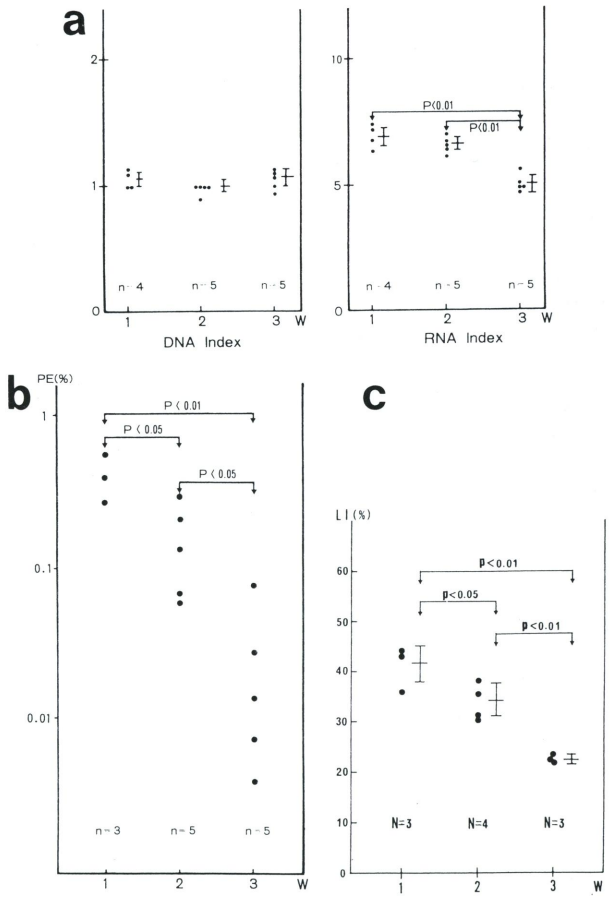

Fig. 4. Changes in DNA-RNA indices (a), plating efficiency (b) and BrdUrd labeling index (c) at 1, 2 and 3 weeks after transplantation of Ah109A cells.

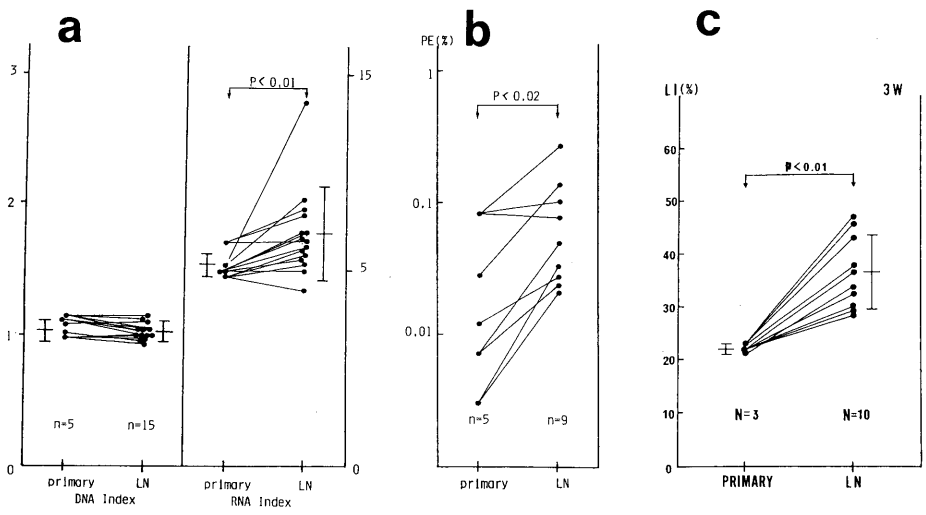

Fig. 5. Comparisons of DNA-RNA indices (a), plating efficiency (b) and BrdUrd labeling index (c) between the primary lesion and the metastatic lymph node in AH109A transplanted Donryu rats.

tion, was studied, PE was likewise found to fall off significantly over time (Fig. 4b). The BrdUrd labeling index also fell off significantly over time after transplantation and fell off markedly at the third week (Fig. 4c).

b) Cell proliferative activity of the primary lesion and the metastatic lymph node

The cell proliferative activity of the primary lesion and the metastatic lymph node was studied in rats three weeks (21 days) after transplantation. When the DNA-RNA indices were examined, the DNA index was about 1.0, there being no difference between the primary lesion and the metastatic lymph node. However, the RNA index showed significantly higher values in the metastatic lymph node than primary lesion ($p < 0.01$) (Fig. 5a). Colony formation (plating efficiency), albeit with some variations, showed significantly high values ($p < 0.02$) in the metastatic lymph node when the primary lesion was compared with the metastatic lymph node (Fig. 5b). In terms of the BrdUrd labeling index, LI increased significantly ($p < 0.01$) in the meatstatic lymph node (Fig. 5c), showing that the cancer cell proliferative activity is higher in the metastatic lymph node.

c) The size of the primary lesion and the metastatic lymph node and cell proliferative activity

The correlation between the size of the primary lesion and the metastatic lymph node on the one hand and the RNA index, plating efficiency, and BrdUrd labeling index on the other was studied. The RNA index at $R = -0.71$ and the labeling index at $R = -0.72$ showed a negative correlation with the size of the primary lesion and the metastatic lymph node. The plating efficiency also showed a similar trend (Fig. 6).

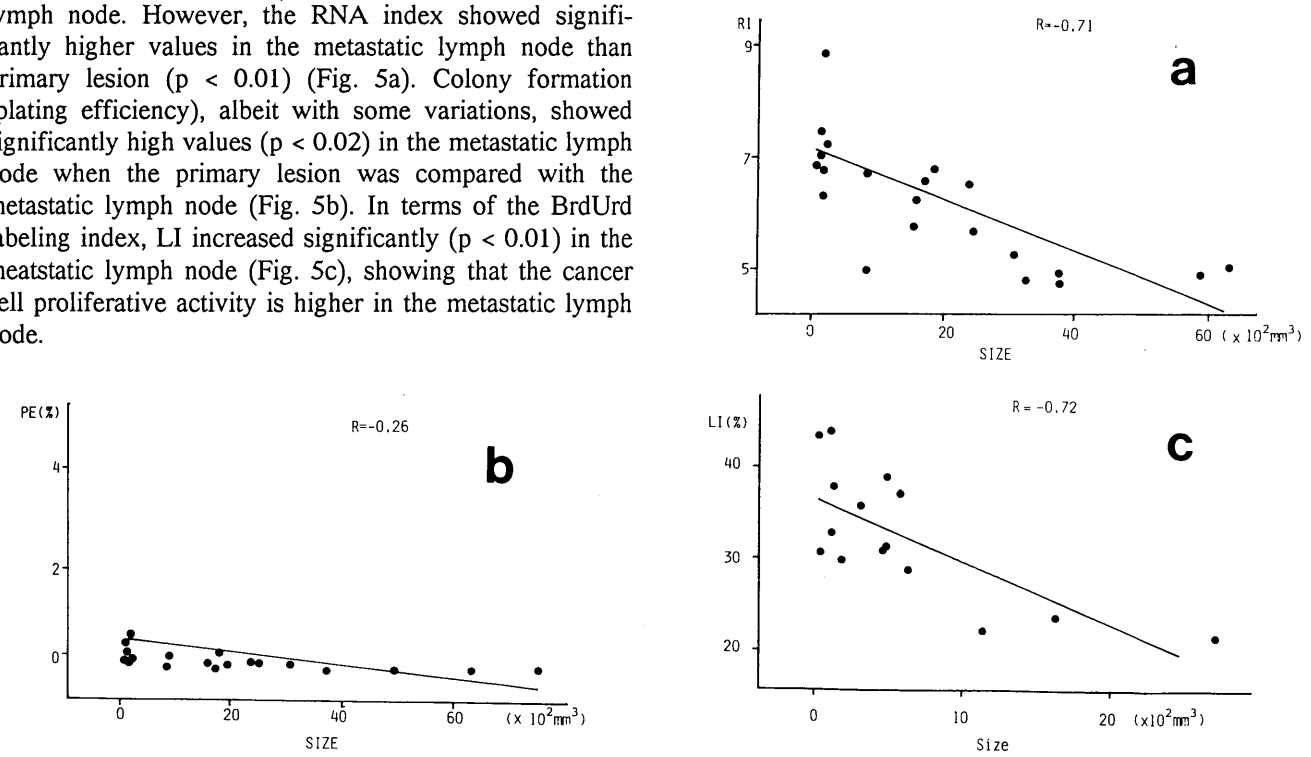

Fig. 6. Correlations between three proliferation parameters and tumor size: (a) :RNA index. (b) :plating effciency, (c): BrdUed labeling index. Corretation coefficient is -0.71 (a), -0.26 (b) and -0.72 (c) respectively.

(2) Experiment with human gastric cancer

The DNA-RNA indices for the primary lesion and the metastatic lymph node were studied in 19 cases of human gastric cancer. The DNA index showed no significant difference between the primary lesion and the metastatic lymph node in 17 out of 19 cases. The remaining two cases showed an increase in DI from diploidy to teraploidy and from triploidy to hypotetraploidy. The RNA index, an indicator for cell proliferative activity, increased significantly ($p < 0.01$) in the metastatic lymph node when the primary lesion was compared with the metastatic lymph node, albeit with a difference in the RI value (Fig. 7).

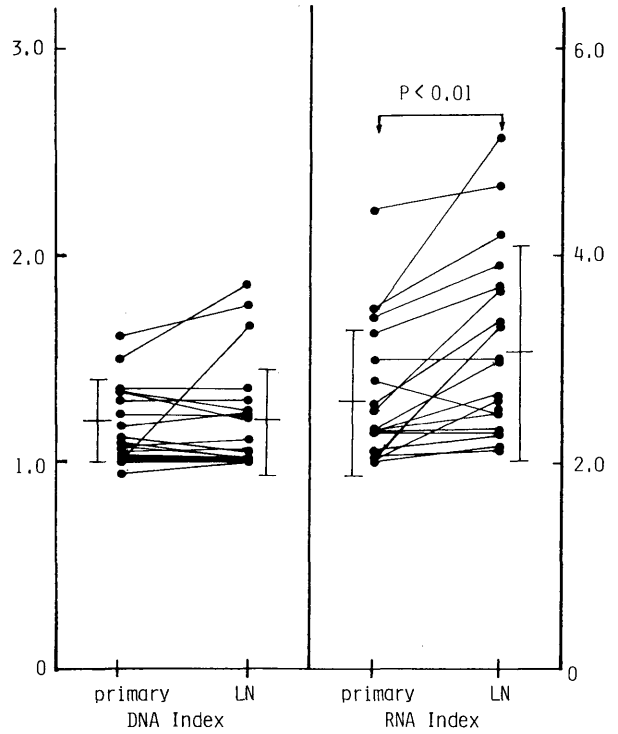

Fig. 7. Comparison of DNA-RNA indices between the primary lesion and the metastatic lymph node in human gastric cancers. N = 19.

Discussion

Differences in the proliferative activity of tumor cells between the primary lesion and the metastatic lymph node were studied with cellular RNA content, colony formation assay and BrdUrd labeling index as the indicators. Differences in the cell composition were also studied with DNA ploidy as the indicator. The cellular RNA content is considered one of the indicators for cell proliferative activity. It decreases when shift is made from the exponential phase to the plateau phase in cultured cells (15). The DNA-RNA content can easily be measured simutaneously and rapidly by flow cytometry using acridine orange staining (10). A more objective evaluation can be made by calculating the DNA-RNA indices.

Andreef et al. (11) studied the relationship between the RNA index and the prognosis of leukemia, reporting that the higher the RI is, the higher is the risk of recurrence. Yamaoka (12) has reported the relationship between the RI and the prognosis in lung cancer. However, this RI varies greatly with cells. In leukemic cells, an RI equivalent to 1/10 of Yamaoka's RI was used. And the RI was higher in DNA aneuploidy than in DNA diploidy. So, it appears preferable for the DNA ploidy levels to be almost equivalent if a more exact evaluation is to be made. AO staining is considered a useful technique as described above, but it is not used frequently, partly because the procedure is somewhat difficult.

Tumor cells are not always a homogenous cell population. As a method for calculating the ratio of cells which exist in this cell population and undergo mitosis for proliferation, the colony formation assay is available. Regarding these cells as the stem cells, Hamberger et al. are using this assay in an anti-tumor chemosensitivity test (16). The colony formation assay is able to evaluate the cell proliferative activity in the different way from the evaluation using the RNA content and BrdUrd labeling index. The other indicator for cell proliferative activity is direct evaluation of DNA synthesis. This has been perfomed by the radioisotope technique using ^{3}H-thymidine. Recently, a monocloanl antibody against BrdUrd, an analogue of thymidine (13) has been developed, which (17) has made it possible to evaluate cells undertaking DNA synthesis easily and rapidly. It is thought that the RNA content, colony formation assay and BrdUrd labeling index each is able to evaluate a different cell proliferative activity. Therefore, these three indicators were selected as markers for cell proliferation in the present study.

The correlation between the length of time after transplantation and the cell proliferative activity was evaluated. The RNA index, plating efficiency and BrdUrd labeling index fell off over time one, two and three weeks after transplantation. Rats die of tumors about 30 days after the transplantation of 1×10^6 cells of AH109A tumor. If the points in time of one, two and three weeks after cell transplantation are considered to be a yardstick for the degree of progression, these correspond to the early, middle and terminal stages respectively. So the cell proliferative activity of the primary lesion declined slowly with progression of the tumor. In clinical studies of solid cancer, it has been reported that the cell proliferative activity increases with progressin of the tumor (12). In examination of the RNA content in gastric cancer, however, the proliferative activity appeared to decline with progression of the tumor. That is probably because cancer is detected early in gastric cancer, while cancer is already advanced despite its small size at the time of detection in other types of cancers.

Whereas the entire tumor was evaluated as a sample in the rats, the tumor mass free of necrosis was evaluated in the clinical studies of cancer.

If the tumor mass is evaluated as a whole, the environment of tumor growth, such as the blood flow, changes with progression of the tumor to cause central necrosis, and the proliferative activity of the tumor declines (18) (19). This is substantiated by the fact that the RNA index and BrdUrd labeling index showed a negative correlation with the tumor size.

When comparison was made between in cell pralif-erative activity the primary lesion and the metastatic lymph node, all three parameters revealed significantly higher values in the metastatic lymph node than in the primary lesion.

Macroscopically evident metastasis occurs after two weeks in this kind of transplanted tumor, and metastasis arises naturally from the primary lesion. Therefore the metastatic lesion is at an earlier stage of tumor development compared with the primary lesion. Viewed from the tumor growth curve, the primary lesion is the plateau phase at this point in time, while the metastatic lesion is still in the exponential phase and therefore is high in cell proliferative activity.

The lymph node is an important site for the immune response of the tumor-bearing host. In metastasis, it may work in an antitumor manner to suppress the proliferation of tumor cells. In this respect, Fisher et al. (2) have observed the anti-tumor ability of the local lymph nodes in a study using experimentally transplanted tumors. However, many investigators support the view that the local lymph node plays an important role only in the stage of recognizing antigens early after transplantation of the tumor and that the anti-tumor ability of the lymph node disappears with growth of the tumor (3) (4) (5).

According to studies on the local lymph node of patients with carcinomas of various organs, many results indicate that the local lymph node has only low anti-tumor ability (7) (8), although there is a report (6) that it shows potent activity as compared with peripheral lymphocytes. Therefore, overt metastatic lymph node in the present study revealed no anti-tumor ability and showed an increase in proliferative activity.

Whether or not cells that constitute the primary lesion and the metastatic lesion are identical was studied with respect to DNA ploidy. The DNA index in rats was about 1.0 for both the primary lesion and the metastatic lesion; both were at the same ploidy level, there being no difference between the two. In the cases of gastric cancer too, they were at the same ploidy level except for two cases in which the ploidy changed from diploidy to triploidy and from triploidy to hypotetraploidy.

From the findings above, it is surmised that tumor cells in the metastatic lymph node consist mostly of the same constituent cells as in the primary lesion and that metastasis by cells different from the primary tumor cells found in the DNA histogram is rare. In this respect, Frankfurt et al. (20) examined DNA ploidy in the primary lesion and the metastatic lesion of 15 cases of various human solid tumors. They reported that 12 cases showed almost the same DNA index, maintaining that both lesions are of the same stemline in many cases. Auer et al. (21) have also reported that the histogram was identical for the primary lesion and metastatic lesion in 17 out of 18 cases when the lymph node was induded on autopsy at the time of diagnosis by cytophotometry in human breast cancer.

On the other hand, tumors are of many kinds, and multiclones have been reported in DNA ploidy particularly in advanced cancer (20) (22). It was taken into consideration that the clone of cells failed to be detected in the primary lesion in cases where the ploidy pattern of the metastasis was not identical to that of the primary site. The clone in the primary lesion may play a leading role in metastasis.

Olszewski et al. (23) calculated the S phase fraction from the DNA histogram for the primary lesion and the metastatic lymph node of breast cancer. When compared between the two, they reported that there were more S phase cells in the metastatic lymph node than in the primary lesion with ER negative.

With this method, however the more normal cells are mixed in, the more the results tend to be inaccurate. As a result the S phase is calculated as being low in diploid tumors. This method is therefore considered inappropriate for lymphatic metastatic lesions which contain many lymphocytes. Therefore it was not used in the present study. In contrast, measurement of the RNA content using AO staining is able to distinguish tumor cells easily, making it possible to measure the proliferative activity more exactly.

The cell proliferative activity was studied using the RNA content in human gastric cancer. Since the BrdUrd assay is complex and in vivo administration poses a problem, the RNA content measurement using acridine orange staining was selected. As a result, it was seen that the proliferative activity of tumor cell is higher in the metastatic lymph node than in the primary lesion even in the case of clinical solid cancer as shown by the DNA and RNA indices.

Kanzawa (24) has reported that the cultrue success rate and the result of the colony formation assay were almost equivalent with the primary and metastatic lesions.

It is suggested that cells in the metastatic lymph node and hematogenous metastasis reveal a high proliferative activity inasmuch as the anti-tumor activity of the lymph node is low. The fact that the cell proliferative activity in the metastatic lesion is higher than in the primary lesion possibly shows that the metastatic lesion is playing a more important role in the progression of the tumor. On the other hand, this fact may indicate that there are many cycling cells in the metastatic lesion. It is therefore possible that there are more cells sensitive to chemotherapeutic agents

designed mainly to inhibit DNA synthesis in the metastatic lesion than in the primary lesion (25).

It is also thought that the primary lesion has many noncycling cells or quiescent cells because of its cell environment and shows resistance to chemotherapy and radiotherapy. Therefore, it is recommended that reduction surgery should be done on the primary lesion, followed by chemotherapy and radiation therapy which are more sensitive for cycling cells, in cases of tumors having metastatic lesions.

In gastric cancer massive lymph node involvement and lymph node recurrence after surgery are often encountered clinically. Especially para-aortic lymph node has been noted recently. It is reported that dissection of evident para-aortic lymph nodes has little advantage on survival rates of gastric cancer patients. (26) Chemotherapy after the proper reduction of the primary tumor could be more effective on the metastatic lymph node in thses cases. Some papers reported the lymph node was more sensitive to antitumor agents than the primary lesion. (27) (28).

The proliferative activity is found to be higher in the metastatic lymph node than in the primary lesion. Therefore, the effects of anti-cancer drugs are expected to be more powerful on the metastatic lymph node than the primary lesion.

The present study could serve as a theoretical basis for reduction surgery on the primary lesion followed by chemotherapy and radiotherapy on secondary lesions.

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