Regional Lymphadenectomy and Tumor Curability in C3H/He Mice Transplanted with MH 134

MASAKAZU SAKITA, YUKINOBU NISHIMURA, ATSUSHI TAKENAKA, OSAMU KOJIMA, BUNZO NISHIOKA, YOSHIHIRO FUJITA and SUSUMU MAJIMA

The First Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto 602

SAKITA, M., NISHIMURA, Y., TAKENAKA, A., KOJIMA, O., NISHIOKA, B., FUJITA, Y. and MAJIMA, S. *Regional Lymphadenectomy and Tumor Curability in C3H/He Mice Transplanted with MH 134*. Tohoku J. exp. Med., 1979, 127 (2), 133-141 — In order to elucidate the effect of surgical removal of the regional nodes on the immunological defence of the host against the tumor, as well as on the cure rate of C3H/He mice transplanted with MH 134, three separate experiments were carried out, and the following results were obtained: 1) The cure rates of mice treated by removal of the primary tumor with regional lymph nodes were significantly lower than that of mice treated by simple amputation without regional lymphadenectomy. 2) It was demonstrated by a bioassay of the regional node that viable tumor cells presented in the nodes prior to the operation disappeared after removal of the primary tumor, whereas in ^{60}Co irradiated mice prior to the tumor inoculation, such phenomena were not observed. 3) Observation utilizing in vivo neutralization revealed that regional lymph node cells were capable of interfering with growth of tumor cells. These findings suggest that removal of the regional nodes impairs the restraint to the growth of residual tumor after the removal of the primary tumor, and this is probably due to a derangement of the immunologic response of the host. ——— regional lymph node; immunological defence of the host; effect of regionallymphadenectomy

Because of the high incidence of microscopic cancer involvement in clinically negative nodes, it has been generally acceptable in a radical operation for cancer to excise the regional lymph nodes along with the primary tumor, regardless of their cancer involvement. As a result, widespread application of this policy in cancer surgery has resulted in a significant percentage of patients who presumably were not helped by such an extended approach because the lymphadenectomy specimen contained no histologic evidence of metastatic tumor.

In the recent past, it was believed that this prophylactic lymphadenectomy had no deleterious effects on the host undergoing this therapeutic regimen. In as early as 1965, however, Crile proposed, basing his opinion on experimental data as well as on surgical practices, that retention of regional lymph nodes, grossly

Received for publication, May 4, 1978.

A part of this work was presented at the 35th Annual Meeting of the Japanese Cancer Association, Tokyo, October, 1976. This work was supported in part by the grant for Cancer Research from the Ministry of Health and Welfare, Japan.

uninvolved with the tumor, may be selectively important in maintaining a degree of systemic immunity against further growth of metastatic microfoci or incisional recurrences from operative wound seeding. Thereafter, it has been demonstrated in animal experiments that regional lymph nodes play an important role in the maintenance as well as in the initiation of the immunological defence of the host against the tumor, and that their removal impairs this immune response of the host (Fisher and Fisher 1971, 1972; Perez et al. 1973, 1975; Fisher et al. 1974; Goldfalb and Hardy 1975; Pendergrast et al. 1976). Moreover, some authors (Deodhar et al. 1972; Fisher et al. 1973; Ellis et al. 1975) have shown that in breast-cancer patients the axillary lymph nodes have a definite activity in vitro against the presence of the primary tumor. This evidence has important significance for clinical cancer surgery and challenges the classic concept of en bloc resection of the primary tumor and the regional lymphatics.

The experiments reported here were designed to examine the effect of surgical removal of the regional lymph nodes on the cure of C3H mice transplanted with MH 134, and its effects on the immunological defence of the host against the tumor are discussed from a surgical viewpoint.

MATERIALS AND METHODS

Tumor-host system

Inbred, 8–12 week old, male C3H/He mice were employed as the host for tumor inoculation. They were maintained in individual cages and fed standard laboratory chow and tap water ad lib. The tumor used was mouse ascites hepatoma 134 (MH 134) of C3H origin which has been transferred in an ascitic form through C3H/He mice. Cell suspensions were withdrawn from their ascites as the occasion demanded and adjusted to the desirable concentration. In this series of experiments, 10^5 tumor cells/0.05 ml were inoculated exactly to the subcutis of the right foot pads of the mice using a 27 gauge needle. Using this tumor-host system, three separate experiments were carried out in the present study.

Experiment I

To examine the effect of surgical removal of the regional lymph nodes on survival of tumor-bearing mice, animals were put into 4 random groups. Those in Group I served as untreated control animals without any surgical procedures after tumor inoculation. Those in Group II or III were treated with simple or radical amputation of the tumor-bearing leg on the 9th day following tumor inoculation. In the simple amputation performed in Group II, the tumor-bearing leg was removed at the knee so as to leave the popliteal nodes intact. In the radical amputation performed in Group III, removal of the ipsilateral popliteal, and inguinal nodes along with the tumor-bearing leg was carried out. In Group IV mice, a single dose of 350 rads of ^{60}Co was irradiated to the whole body 24 hr prior to tumor inoculation, and simple amputation of the tumor-bearing leg was performed 9 days after tumor inoculation.

All animals in each group were observed at least 100 days or until death. They were carefully examined either macroscopically or microscopically to see whether they were free from tumor growth or with metastases. "Cure" represented survival of the animals to day 100 without any evidence of tumor growth or metastases.

Experiment II (biological detection of tumor cells in regional lymph nodes)

In order to examine the distribution of viable tumor cells in the regional lymph nodes at various intervals before and after removal of the tumor-bearing leg, the following experi-

ment was performed in 2 groups of mice with and without whole-body irradiation of a single dose of ^{60}Co 24 hr prior to tumor inoculation.

In both groups of mice, 10^5 tumor cells were inoculated in the right foot pad, and simple amputation of the tumor-bearing leg was done on the 9th day after tumor inoculation. Extirpation of the right popliteal nodes was carried out immediately prior to or 1, 2, 4, 6 days after removal of the tumor-bearing leg, that is, at the 9, 10, 11, 13 and 15th day following tumor inoculation.

The extirpated lymph nodes were minced with scissors in a petri dish, the mash suspended in 0.1 ml of physiologic saline, and injected intraperitoneally into normal mice. Seven to ten mice were assayed each time. In each instance, a recipient mouse was used for each donor, which allowed the identification of viable tumor cells in the lymph nodes in treated mice with death from ascitic tumor.

Experiment III (in vivo neutralization)

Using lymphoid tissues obtained from the mice one day after removal of the tumor-bearing leg which had been present for 9 days, an in vivo neutralization test was carried out. The lymphocytes used were obtained from the following sources: 1) regional lymph nodes (RN), i.e. ipsilateral popliteal and inguinal nodes, 2) distant lymph nodes (DN), i.e. contralateral popliteal and inguinal nodes, and 3) spleen, referred to as central lymph nodes (CN). Lymph nodes and spleen were removed from 20–30 ether-anesthetized mice using sterile precautions, and placed in Hank's solution. They were teased apart to free cells, and these cell suspensions were passed through a nylon sieve into fresh Hank's solution to prepare a single cell suspension. The cells were counted in a hemocytometer and diluted to contain 60×10^6 viable cells per ml. Tumor cells were also counted and diluted to contain 1×10^5 viable cells per ml. Lymphocyte and tumor cell suspensions were mixed so that each recipient received an inoculum of 1×10^4 tumor cells and 6×10^6 lymphocytes in 0.2 ml. The resulting suspension was injected subcutaneously into the right hind legs of normal mice.

Animals were put into four random groups (10 mice per group): 1) those injected with tumor cells alone (control), 2) tumor cells+RN-cells, 3) tumor cells+DN-cells, and 4) tumor cells+spleen cells (CN-cells). Mice in each group were examined frequently to measure the thickness of their right hind legs where the mixed suspensions were inoculated. The experiment was terminated 40 days following tumor inoculation. Data were analyzed by Student's *t*-test for significance at a level of 0.05.

RESULTS

Effect of removal of the regional lymph nodes on cure in tumor-bearing mice

The results of Experiment I are summarized in Table 1, and the survival curves of the four groups are illustrated in Fig. 2. In the control group (Group I), all the untreated mice developed marked pulmonary metastases as well as lymph node metastases in the ipsilateral popliteal, inguinal and para-aortic regions (Fig. 1), and died from tumors within 58 days following the tumor inoculation. Their mean survival period was 48.4 ± 7.1 days.

Among the three experimental groups, the simple amputation group without regional lymphadenectomy (Group II) exhibited a cure rate of 80 percent while a cure rate in the radical amputation group with regional lymphadenectomy (Group III) was only 45 percent. In addition, when the mice received whole-body irradiation of ^{60}Co (350 rads single dose) 24 hr before tumor inoculation (Group IV), only 30 percent of the mice were cured after simple amputation of the tumor-bearing leg. The difference in the cure rate between Group II and Group III or IV was

TABLE 1. Cure rates and mean survival times of dead mice in the four groups (Exp. I)

Group	Cure rate	Mean survival of dead mice (days $\pm$ S.D.)
	%	
Group I (untreated control)	0 (0/20)*	48.4 $\pm$ 7.1
Group II (simple amputation on day 9)	80 (16/20)	48.5 $\pm$ 10.2
Group III (radical amputation on day 9)	45 (9/20)	44.4 $\pm$ 7.1
Group IV (^{60}Co + simple amputation on day 9)	30 (3/10)	39.1 $\pm$ 10.8

* Number of cured mice/number of mice examined.

Fig. 1. Untreated mice developing marked pulmonary metastases (upper) as well as lymph node metastases in the ipsilateral popliteal, inguinal and para-aortic region (lower).

Fig. 2. Survival curves of the four groups in Experiment I.

statistically significant ($p < 0.05$). All the dead mice in the experimental groups had similar lung and/or lymph node metastases as those observed in the untreated mice. In contrast, none of the cured mice showed evidence of tumor regrowth or metastases following the operation.

Bioassay of the regional lymph nodes before and after removal of the primary tumor

In Experiment II, biological detection of viable tumor cells migrating to the regional lymph nodes from the primary tumor was carried out at various times before and after amputation of the tumor-bearing leg using a bioassay of the right popliteal lymph nodes. The results are shown in Table 2.

TABLE 2. *Biologic detection of tumor cells in popliteal node of non-irradiated or ^{60}Co irradiated mice at various intervals following amputation of tumor bearing leg*

Days after amputation	Non-irradiated mice	^{60}Co irradiated mice
	%	%
0	7/8* (88)	9/9* (100)
1	0/8 (0)	4/10 (40)
2	0/8 (0)	4/9 (44)
4	0/8 (0)	7/10 (70)
6	0/7 (0)	6/8 (75)

* Number of positive nodes/number of nodes examined.

In the non-irradiated mice, a bioassay performed immediately prior to the operation (on day 9 following tumor inoculation) demonstrated the presence of viable tumor cells in the right popliteal nodes of 88 percent of the mice, whereas, when the tumor-bearing leg was removed, bioassay performed 1, 2, 4 or 6 days after the operation never exhibited the presence of viable tumor cells in the right popliteal nodes of any of the mice. On the other hand, it was noted that in the irradiated mice shortly before the operation viable tumor cells were detected in the regional lymph nodes of all the animals, and even after the operation the lymph nodes of 40-75 percent of the mice remained positive. These differences in the positive rate after the operation were statistically significant between the 2 groups ($p < 0.05$).

In vivo neutralization experiment

It has been indicated recently by some investigators (Fisher et al. 1974; Perez et al. 1975) that regional lymph nodes (RN) cells are capable of destroying or inhibiting the growth of tumor cells which lodge in them, and that such activity is limited to RN-cells. Consequently, an in vivo neutralization experiment was carried out to determine whether this phenomenon could be observed also in the tumor-host system employed in the present study. The regional lymph nodes, distant lymph nodes (DN), and spleen (CN) used for this experiment were obtained from the mice that harbored the tumors for 9 days. The results of this

experiment are shown in Table 3 and Fig. 3. The growth curves (Fig. 3) of tumors which developed in the four groups of mice injected with tumor cells with or without various types of lymph nodes cells were made by plotting the average thickness of tumor-bearing legs in 10 mice, because the size of a growing tumor was most accurately evaluated by measuring the thickness of the hind-leg at the site of tumor inoculation.

Fig. 3. In vivo neutralization of tumor cells by lymphoid cells from the mice a day after tumor removal. ○—○, tumor cells+regional lymph node cells; ▲—▲, tumor cells+distant lymph node cells; △—△, tumor cells+spleen cells; ●—●, control.

TABLE 3. Incidence of mice with tumors and their average survival times (in vivo neutralization experiment)

	Number of mice	Number of mice with tumor	Average survival days (days±s.d.)
		(%)	
RN	10	6 (60)	29.6±3.9
DN	10	10 (100)	20.1±2.5
CN	10	10 (100)	25.2±3.6
Control	10	10 (100)	24.6±3.8

In the control group of mice injected with tumor cells alone, all the mice died from progressively growing tumors, with a mean survival of 24.6 ± 3.8 days (Table 3). Also, in the DN-group or the CN-group of mice undergoing injection of tumor cells mixed with either DN-cells or CN-cells, all mice died from tumors, and their mean survival was 20.1 ± 2.5 days (DN-group) or 25.2 ± 3.6 days (CN-group). In contrast, in the RN-group of mice injected with tumor cells and RN-cells, 4 of 10 mice had no tumor development and the remaining 6 mice, or 60%, died from tumors, with a mean survival of 29.6 ± 3.9 days. Thus, the incidence of tumor growth in the RN-group of mice was significantly lower than those in the other three groups ($p < 0.05$). Furthermore, the growth of tumors developing in the RN-

group was almost completely inhibited for 2 weeks after inoculation of the mixed suspension, and the survival period of the mice with tumor development was significantly prolonged as compared with those of the other groups. As a result of this *in vivo* neutralization, it was found that regional lymph nodes cells in the tumor-host system used in the present study were capable of interfering with the growth of tumor cells and that neither the distant lymph nodes cells nor the spleen cells displayed this characteristic.

DISCUSSION

The regional lymph nodes, which at one time were presumed to be a mechanical barrier against the dissemination of tumor cells, are more recently regarded as an essential component of the host's immune response against a tumor (Futrell and Pories 1975). Indeed, abundant evidence, mainly from animal experiments, has been accumulated to support the concept that the immunologic response of the host, particularly of the regional lymph nodes, plays an important role in preventing tumor dissemination or contributes to the cure of tumors treated by conventional surgical procedures. Some authors (Prehn 1965; Crile 1965, 1968; Fisher et al. 1972) stress the importance of preserving the regional lymph nodes unless invaded by tumor, since their removal may be detrimental to the ability of the host to control tumor growth and dissemination.

In the present experiments, a significant decrease (45 percent) in the cure rate was noted in the group of mice subjected to radical amputation of a tumor-bearing leg with regional lymphadenectomy on the 9th day following tumor inoculation, as compared to that (80 percent) noted in the group of mice treated by simple amputation without regional lymphadenectomy. In addition, the survival periods of the mice were significantly shorter in the radical amputation group than in the simple amputation group. Since MH 134 used in the present experiments characteristically metastasizes the lymph nodes and the lung at a relatively early stage after tumor inoculation (Sato 1959; Hashimoto 1964), these data strongly suggest that regional lymphadenectomy impairs the ability of the mice to prevent the proliferation of tumor cells which had disseminated to these organs before resection of the primary tumor.

On the other hand, a bioassay of the regional lymph nodes documented that at the time of amputation of the tumor-bearing leg (9th day after tumor inoculation), approximately 90 percent of the mice had enough viable tumor cells in their right popliteal nodes to kill the recipient mice. However, in bioassays carried out during 6 days following the operation, these lymph nodes became negative in all the mice. Furthermore, observations utilizing *in vivo* neutralization have revealed that at the time of amputation of the tumor-bearing leg, regional lymph node cells were capable of interfering with the growth of tumor cells. However, neither distant lymph nodes cells nor spleen cells ever fully displayed their neutralizing capabilities. These findings are compatible with those of Fisher et al. (1974) who have indicated that regional lymph nodes are unique from the rest of the lymphoreticular system

in so far as the immunologic response of a host to its tumor is concerned. Our observations also suggest that viable tumor cells disseminating to the regional lymph nodes may be destroyed in these structures, and that the findings of negative lymph nodes observed in their bioassays may be the result of such a circumstance.

The results in the group of mice treated with whole-body irradiation of ^{60}Co prior to tumor inoculation were considerably different from those in the non-irradiated mice described above. The cure rate of mice treated by simple amputation of a tumor-bearing leg without regional lymphadenectomy was significantly lower in the group of irradiated mice (30 percent) than in the group of non-irradiated mice (80 percent). Also in bioassays of the regional lymph nodes, performed after amputation of the tumor-bearing leg, all of the non-irradiated mice showed negative lymph nodes while 40–75 percent of the irradiated mice had positive lymph nodes. These differences between the two groups may be due to the suppression of a cell-mediated immunity by whole-body irradiation of ^{60}Co .

Thus, our bioassay observations and the results of the *in vivo* neutralization experiment, coupled with the marked decrease in the cure rate of lymphadenectomized mice, support the hypothesis that the removal of the regional lymph nodes impairs the restraint to the growth of residual tumor, i.e. metastases, after the removal of the primary tumor, and this is probably due to a derangement of the immunologic response.

In general, it is often difficult to extrapolate to human cancer the results of studies done with animal neoplasms because of the difference in biological behavior. Accordingly, the present observation may not occur so strikingly in human cancer. Particularly, the relatively short duration of the presence of the "primary" tumor in the present experiments may suggest that the results reported here cannot be compared with those in humans where tumors may be present for a protracted period, allowing for the development of systemic immunity. However, some clinical observations suggest that the removal of non-metastatic regional lymph nodes may result in a decreased cure rate, which has been particularly emphasized by Crile (1965) in carcinoma of the breast. Further knowledge about the natural history of human neoplasms, and their chronological pattern of dissemination, may demonstrate that the experimental data presented here are clinically relevant.

References

- 1) Crile, G., Jr. (1965) Rationale of simple mastectomy without radiation for clinical stage I cancer of the breast. *Surg. Gynec. Obstet.*, **120**, 975–982.
- 2) Crile, G., Jr. (1968) Effect on metastasis of removing or irradiating regional nodes of mice. *Surg. Gynec. Obstet.*, **126**, 1270–1272.
- 3) Deodhar, S., Crile, G., Jr. & Esselstyn, C. (1972) Study of the tumor cell lymphocyte interaction in patients with breast cancer. *Cancer (Philad.)*, **20**, 1321–1325.
- 4) Ellis, R.J., Wernick, G., Zabriskie, J.B. & Goldman, L.I. (1975) Immunologic competence of regional lymph node in patients with breast cancer. *Cancer (Philad.)*, **35**, 655–659.
- 5) Fisher, B. & Fisher E.R. (1971) Studies concerning the regional lymph node in

- cancer. I. Initiation of immunity. *Cancer (Philad.)*, **27**, 1001-1004.
- 6) Fisher, B. & Fisher, E.R. (1972) Studies concerning the regional lymph node in cancer. II. Maintenance of immunity. *Cancer (Philad.)*, **29**, 1496-1501.
 - 7) Fisher, E.R., Saffer, E. & Fisher, B. (1973) Studies concerning the regional lymph node in cancer. VI. Correlation of lymphocyte transformation of regional node cells and some histopathologic discriminants. *Cancer (Philad.)*, **32**, 104-111.
 - 8) Fisher, B. Saffer, E. & Fisher, E.R. (1974) Studies concerning the regional lymph node in cancer. IV. Tumor inhibition by regional lymph node cells. *Cancer (Philad.)*, **33**, 631-636.
 - 9) Futrell, J.W. & Pories, W.J. (1975) Physiologic and immunologic considerations of the lymphatic system in tumors and transplants. *Surg. Gynec. Obstet.*, **140**, 273-292.
 - 10) Goldfarb, P.M. & Hardy, M.A. (1975) The immunologic responsiveness of regional lymphocytes in experimental cancer. *Cancer (Philad.)*, **35**, 778-783.
 - 11) Hashimoto, K. (1964) Studies on metastasis of cancer. XVIII. Experiments on lymph nodes metastasis with reference to preventive effect of surgical removal of the regional lymph nodes on recurrence and metastasis. *Sci. Rep. Inst. Tohoku Univ.*, **11**, 406-418.
 - 12) Pendergrast, W.J., Soloway, M.S., Myers, G.H. & Futrell, J.W. (1976) Regional lymphadenectomy and tumor immunity. *Surg. Gynec. Obstet.*, **142**, 385-390.
 - 13) Perez, C.A., Stewart, C.C., Palmer-Hanes, L.A. & Powers, W.E. (1973) Role of regional lymph nodes in cure of murine lymphosarcoma. *Cancer (Philad.)*, **32**, 562-572.
 - 14) Perez, C.A., Stewart, C.C. & Wagner, B. (1975) Regional lymphadenectomy and tumor curability. *Amer. J. Roentgenol.*, **123**, 621-630.
 - 15) Prehn, R.T. (1965) Role of immunity in the biology of cancer. In: *Fifth Nat. Cancer Conf. Proc.*, Philadelphia, J.B. Lippincott Co., pp. 97-104.
 - 16) Sato, H. (1959) Experimental studies on the mechanism of metastasis formation. *Acta path. jap.*, **9**, 685-706.
-