

## **Preoperative Irradiation Combined with Chemotherapy Impairs Healing of Bronchial Anastomosis During the Early Postoperative Period in Rats**

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INOUE, M., OKA, T., SHIMA, Y., SHIRAFUJI, T., SUMIDA, Y., YAMASHITA, H., MURAOKA, M., NAGAYASU, T., AKAMINE, S., SHIMOKAWA, I. and AYABE, H. *Preoperative Irradiation Combined with Chemotherapy Impairs Healing of Bronchial Anastomosis During Early Postoperative Period in Rats.* Tohoku J. Exp. Med., 2003, **199**(1), 1-12 — The effect of preoperative irradiation and antineoplastic agents on healing at the site of bronchial anastomosis was investigated using rats. The bursting pressure in irradiation group and combined irradiation and chemotherapy group was significantly lower than in control and chemotherapy group at day 5 after operation. There was no significant difference in bursting pressure in all groups at day 7. The histologic finding of the anastomosis with H & E stain showed that submucosal connective tissue had not regenerated, and defects were seen in the submucosal tissue in irradiation and combined therapy group at day 3 and day 5. But, the connective tissue had matured in irradiation group at day 7 compared with control group. In conclusion, this study demonstrated that the healing of bronchial anastomosis was markedly delayed in early postoperative days in the rats receiving irradiation and combined therapy. ——— wound healing; bronchial anastomosis; irradiation; chemotherapy; rat  
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In recent years, the use of chemotherapy and radiotherapy preoperatively has become an important form of adjuvant therapy for locally advanced non-small cell lung cancer (NSCLC) (Yashar et al. 1992; Mathisen et al. 1996).

Preoperative chemotherapy and radiotherapy have resulted in improvement and control of micrometastasis from locally advanced NSCLC. On the other hand, bronchoplastic procedure is a valid alternative to pneumonectomy with an

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advantage of preservation of functional lung parenchyma (Gaissert et al. 1996). These procedures have been accepted as curative and safe for lung cancer (Kawahara et al. 1994). Anatomic complications following bronchoplastic procedure are critical and frequently fatal, and the effect of anti-neoplastic agents and thoracic irradiation on healing of bronchial anastomosis remains unclear. Previous experimental studies in rats showed that preoperative cisplatin-etoposide treatment (PE) did not impair wound healing of bronchial anastomosis even in the presence of mild leukopenia and/or after a sufficient interval was allowed for recovery from myelosuppression (Shirafuji et al. 2001).

In the present study, we examined the effects of preoperative thoracic irradiation and concomitant PE on wound healing at the site of bronchial anastomosis in rats.

## MATERIALS AND METHODS

### *Experimental animals*

Male Wistar rats ( $n=126$ , age, 8 weeks) weighing 270 to 300 g (Charles River Japan, Yokohama) were used for assessment of the wound healing. All rats used in this study were maintained in the Animals Center of Biomedical Research, Nagasaki University School of Medicine, and received humane care in compliance with the guidelines of the Animal Care and Use Committee of Nagasaki University. These

procedures conformed to the guidelines established by the National Institutes of Health and published in the Guide for the Care and Use Laboratory animals (NIH Publication No. 85-23).

### *Experimental design*

Rats were divided into two experimental groups ( $n=54$  each), and a control group ( $n=18$ ). Furthermore, within each experiment, rats were divided into three subgroups, each consisting of 18 rats. Experimental groups were as follows: rats of the first group represented the control group, those of the second group received intravenous cisplatin and etoposide (chemotherapy [CT] group), while rats of group 3 received thoracic irradiation consisting of a single dose of 20 Gy (irradiation [IR] group), and rats of group 4 received thoracic irradiation of a single dose of 20 Gy and simultaneous intravenous injection of cisplatin and etoposide (chemotherapy and irradiation [CT+IR] group). Surgery was subsequently performed in all rats. In experiment 1, surgery was performed 3 days after preoperative drug administration and thoracic irradiation have been completed (Fig. 1). In experiment 2, surgery was performed 7 days after preoperative treatments to allow for examination of the effects of recovery of myelosuppression on wound healing. The interval was counted from the last drug administration day or thoracic irradiation day

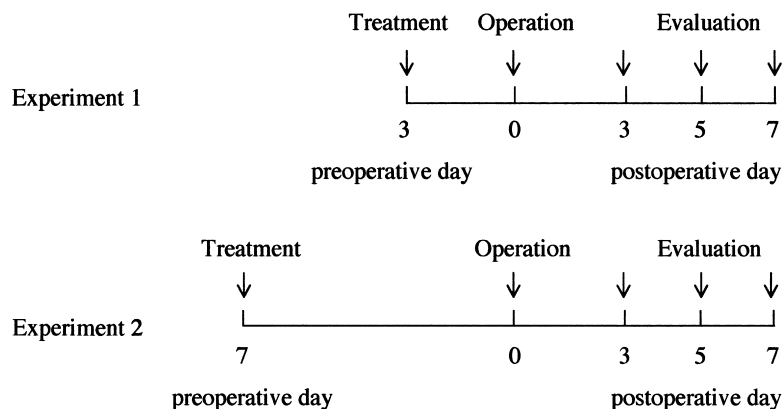


Fig. 1. Experimental design.

to the operation day. All rats were assessed after 3, 5, and 7 days after surgery (see below).

#### *Preoperative drug administration*

The dose of cisplatin was 4 mg/kg and that of etoposide 4.8 mg/kg. Cisplatin was administered via the tail vein on the first day and etoposide was injected on the first, second, and third days. The selected doses were based on previous studies, and represented LD<sub>10</sub> (Karppinen and Myllarniemi 1970; Warram 1975; Ward and Fauvie 1976).

#### *Thoracic irradiation*

Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Irradiation field was determined between the upper and lower edges of the sternal incision. The left and right boundaries of the radiation field were the respective mid-sternal lines. Anatomically, this field included the trachea, left and right main stem bronchi, both lungs, esophagus, heart, thymus, vertebral column and the spinal cord. The rest of the body was shielded with lead during irradiation. Irradiation was performed by means of thermoluminescent dosimeters and film densitometry in separate animals. Irradiation was delivered in a vertical beam from a 250-kVp x-ray unit (EXS-300, Toshiba Corporation, Tokyo). The equipment was operated at 15 mA and 200 kV. Beam was filtered by 0.5 mm Al. The dose rate was 0.85 Gy/min at the focus skin distance of 50 cm.

#### *Operative procedure*

All rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), incubated and ventilated at a tidal volume of 10 ml/kg and respiratory rate of 90 breaths/min using a respirator (SN-480-7, Shimano Corporation, Tokyo). Using clean but nonsterile surgical technique, a 4-cm incision was made on the left chest wall and thoracotomy was performed at the fourth intercostal space. A left main stem bronchial transec-

tion and bronchial end-to-end anastomosis was carried out under a surgical microscope using 8-0 polypropylene sutures (Ethicon, Somerville, NJ, USA). Anastomosis of the cartilaginous and the membranous portions was performed using 6 continuous and 4 interrupted sutures, respectively. The same surgery was performed in the control rats, which did not receive chemotherapy or radiotherapy.

#### *Evaluation*

Body weight was recorded daily. All signs of illness, reaction to treatment, and deaths were recorded during follow-up period. Rats were sacrificed by cardiac puncture at day 3, 5 or 7 after operation ( $n=6$  rats, each). Cardiac blood samples were obtained by open thoracotomy technique and then placed into heparinized syringes. Complete blood counts and albumin contents, total proteins in serum were examined at day 3, day 5 and day 7. Bronchial anastomotic area was harvested at postmortem, and the surrounding tissues adherent to the anastomosis were left untouched microsurgically. The right main stem bronchus and the left main bronchus distal to the anastomotic site were clamped after washing out the intraluminal air. The trachea was connected to an infusion pump and a manometer (RMP-6004S, Nihon Kohden, Tokyo). The pressure was increased with an infusion rate of 0.8 ml/min, which was recorded polygraphically. The bursting pressure was measured and the site of rupture was noted.

If the site of rupture was outside the suture line, the bursting pressure was measured at this point. The anastomotic segment was cleaned from surrounding tissue after assessment of bursting pressure, then the cartilaginous or the membranous portion was dissected. The membranous portion containing the suture line was collected for analysis of hydroxyproline content as a parameter for collagen. The samples were frozen immediately and stored at  $-80^{\circ}\text{C}$ . These were evaporated to dryness in vacuum

for 48 hour, and weighed. The samples were hydrolyzed in 0.5 ml 20% HCl at 110°C for 24 hours, and subsequently evaporated in a rotary vacuum evaporator at 60°C. They were subsequently redissolved in phosphate buffer 1 ml/kg dry weight, and hydroxyproline content was determined using an auto amino acid analyzer (JLC-300, Nippon Denshi, Tokyo).

#### Histopathological examination

For histopathological examination, the cartilaginous portion was fixed in 4% paraformaldehyde in phosphate-buffered saline (4% PFA-PBS, pH 7.4) overnight for hematoxylin-eosin staining and Masson-trichrome staining.

#### Statistical analysis

Group values were expressed as the mean  $\pm$  S.D. Differences between experimental groups and the control were tested for significance with the ranksum two-sample test (Mann-Whitney's U-test) or chi-square test, with significance levels set at 5%.

## RESULTS

### Experiment 1 (preoperative treatment 3 days before surgery)

*General observation.* All animals showed weight loss during postoperative period. Rats of CT+IR group and CT group showed a significant weight loss compared with CONT group and IR group ( $p < 0.05$ ) (Fig. 2). None of the rats had complications arising from anastomotic failure or related to thoracic wound healing.

*Peripheral blood.* Significant leukopenia was noted in CT and CT+IR groups at day 3 compared to the control and IR groups ( $p < 0.05$ ). However, the leukocyte counts recovered gradually, and were already improved at day 7 after surgery (Fig. 3). The platelet counts showed same change as leukocyte counts. There were no significant differences in total protein counts contents among the four groups.

*Bursting pressure.* At postoperative day 3, the bursting pressures were  $201 \pm 80$  mmHg in IR group and  $188 \pm 82$  mmHg in CT+IR group, which were lower than in the control ( $244 \pm 46$  mmHg) and CT ( $275 \pm 69$  mmHg) groups, albeit

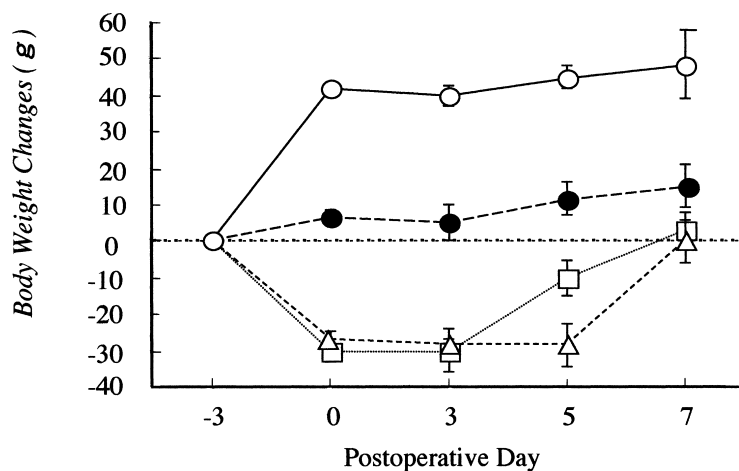


Fig. 2. Body weight changes in experiment 1.

Rats of CT+IR group and CT group showed a significant weight loss compared with CONT group and IR group ( $p < 0.05$ ). CONT, control; CT, rats treated preoperatively with chemotherapy; IR, rats treated preoperatively with 20 Gy irradiation; CT+IR, rats treated preoperatively with chemotherapy and irradiation.

○, CONT; □, CT; △, CT+IR; ●, IR.

insignificantly (Fig. 4). At postoperative day 5, the bursting pressure was significantly lower in IR group ( $430 \pm 257$  mmHg) and CT+IR group ( $433 \pm 227$  mmHg) compared with the control ( $797 \pm 146$  mmHg) and CT ( $798 \pm 106$  mmHg)

groups ( $p < 0.05$ , each). At postoperative day 7, the bursting pressure varied among the groups but this was statistically insignificant (Fig. 2).

*Hydroxyproline content.* At postoperative days 3 and 5, the hydroxyproline contents at the

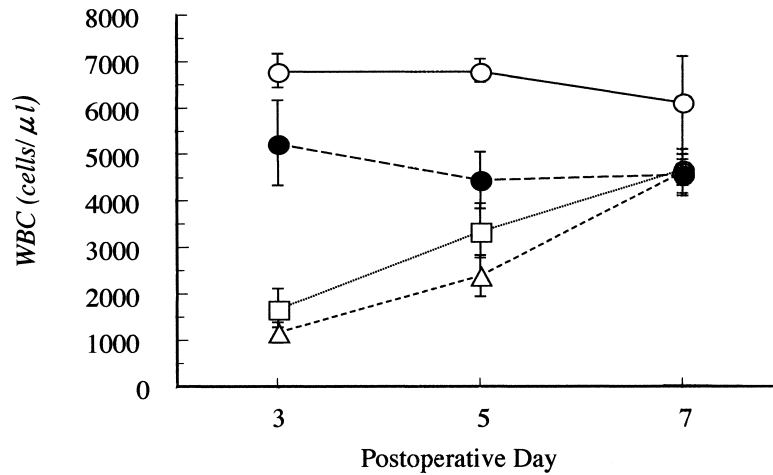


Fig. 3. WBC in experiment 1.

Significant leukopenia was noted in CT and CT+IR groups at day 3 compared to the control and IR groups ( $p < 0.05$ ). However, the leukocyte counts recovered gradually, and were already improved at day 7 after surgery. For abbreviations, see Fig. 2.

○, CONT; □, CT; △, CT+IR; ●, IR.

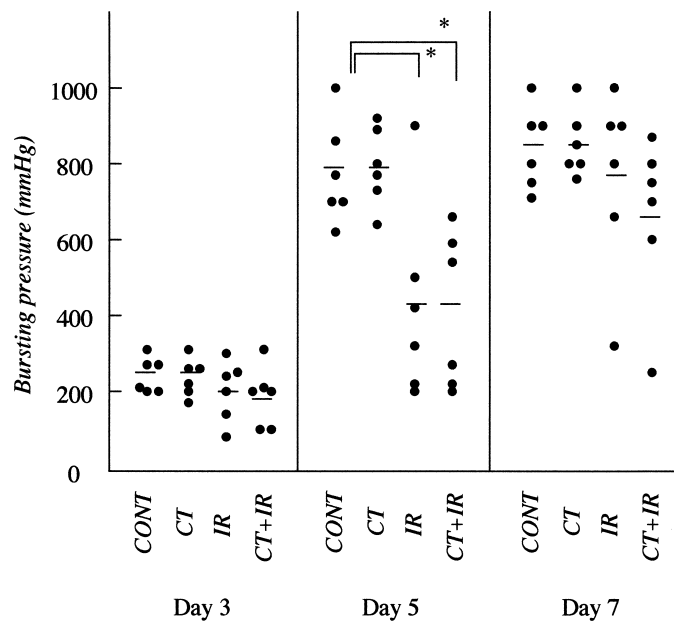


Fig. 4. Anastomotic bursting pressures in experiment 1.

Each point represents a single anastomosis. The horizontal lines represent the mean values. \*The mean values of IR and CT+IR groups were significantly lower than those of the control and CT groups ( $p < 0.05$ ). For abbreviations, see Fig. 2.

○, CONT; □, CT; △, CT+IR; ●, IR.

site of anastomosis were significantly higher in IR and CT+IR groups than in the control and CT groups ( $p < 0.01$ , each) (Table 1). At day 7, there were no significant differences between all groups.

*Histopathological findings.* H & E staining

TABLE 1. *Anastomotic hydroxyproline contents in experiment 1*

Group	Day 3	Day 5	Day 7
CONT	149.8±8.4	168.2±18.3	201.3±31.0
CT	147.8±25.9	180.8±35.8	216.2±25.2
IR	199.7±34.4*	237.2±20.4*	199.2±28.1
CT+IR	217.5±17.3*	242.2±36.8*	233.1±28.2

Anastomotic hydroxyproline contents are expressed mean (n mol/mg dry weight)±s.d.. \* $p < 0.01$ . significantly higher than in CONT group and CT group. For abbreviations, see Fig. 2.

of the tissue sections obtained from the site of anastomosis showed excellent growth of the submucosal connective tissue in the control group at days 3 and 5. In contrast, the submucosal connective tissue showed no signs of regeneration, and defects were seen in the submucosal tissue in IR group at days 3 and 5 (Fig. 6). Mature connective tissue was recognized in IR and CT+IR groups at day 7 compared with the control group (data not shown). Masson-trichrome staining of the anastomotic tissues showed lack of maturity of granulocyte tissue and extracellular matrix in IR and CT+IR groups at days 3 and 5 (data not shown).

*Experiment 2 (preoperative treatment 7 days before surgery)*

*General observation.* All animals showed

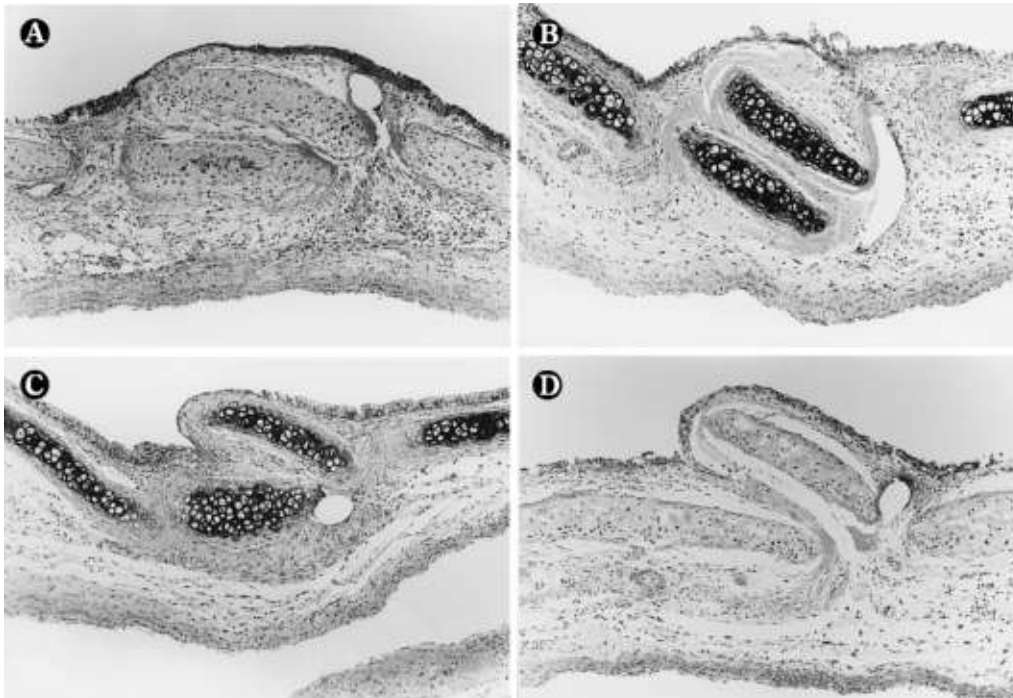


Fig. 5. Histopathological findings in H&E stained sections of rats participating in experiment 1 ( $\times 100$ ).

A: Excellent growth of submucosal connective tissue and mucosal epithelium at postoperative day 3 (control group). B: Excellent growth of submucosal connective tissue and mucosal epithelium at postoperative day 5 (control group). C: The numbers of fibroblasts and macrophages were lower in the radiochemotherapy group compared with the control group at postoperative day 3. D: Note that the connective tissue around tracheal cartilage at the site of anastomosis is thin and edematous at day 5 in a representative rat of the radiochemotherapy group. Note also the defects in the submucosal tissue.

weight loss during the postoperative period. Rats of CT+IR group and CT group showed a significant weight loss compared with CONT group ( $p < 0.05$ ) (Fig. 6). None of the rats had complications arising from anastomotic failure or related to thoracic wound healing.

*Peripheral blood.* At postoperative day 3, leukocyte counts had already improved in IR and IR+CT groups and further increased to normal levels after that day (Fig. 7). The

platelet counts showed same change as leukocyte counts. There were no significant differences in total protein counts contents among the four groups.

*Bursting pressure.* At postoperative day 3, the bursting pressures in IR ( $180 \pm 38$  mmHg) and CT+IR ( $192 \pm 33$  mmHg) groups were lower than in the control ( $244 \pm 47$  mmHg) and CT ( $275 \pm 69$  mmHg) groups, albeit statistically insignificant (Fig. 4). At postoperative day 5,

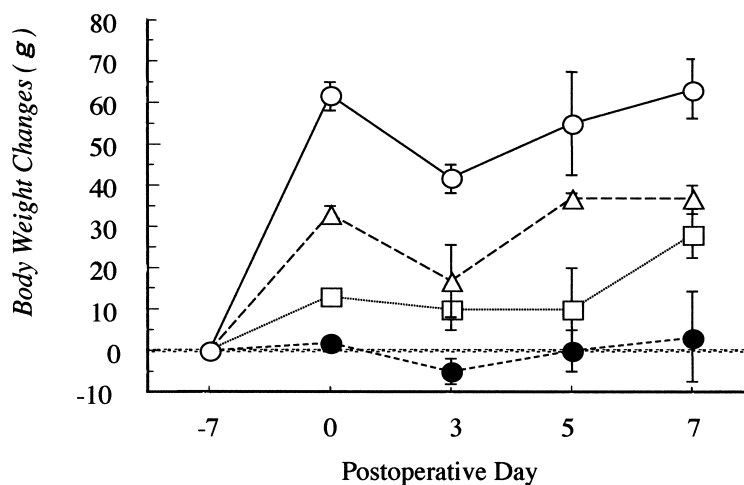


Fig. 6. Body weight changes in experiment 2.

Rats of CT+IR group and CT group showed a significant weight loss compared with CONT group ( $p < 0.05$ ). For abbreviations, see Fig. 2.

○, CONT; □, CT; △, CT+IR; ●, IR.

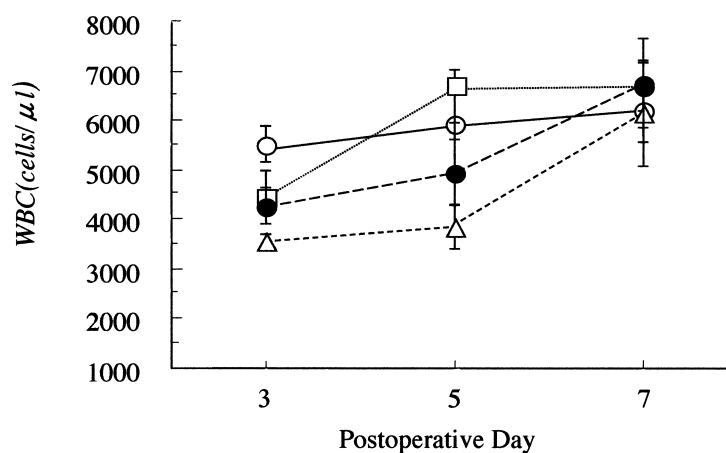


Fig. 7. WBC in experiment 2.

At postoperative day 3, leukocyte counts had already improved in IR and IR+CT groups and further increased to normal levels after that day. For abbreviations, see Fig. 2.

○, CONT; □, CT; △, CT+IR; ●, IR.

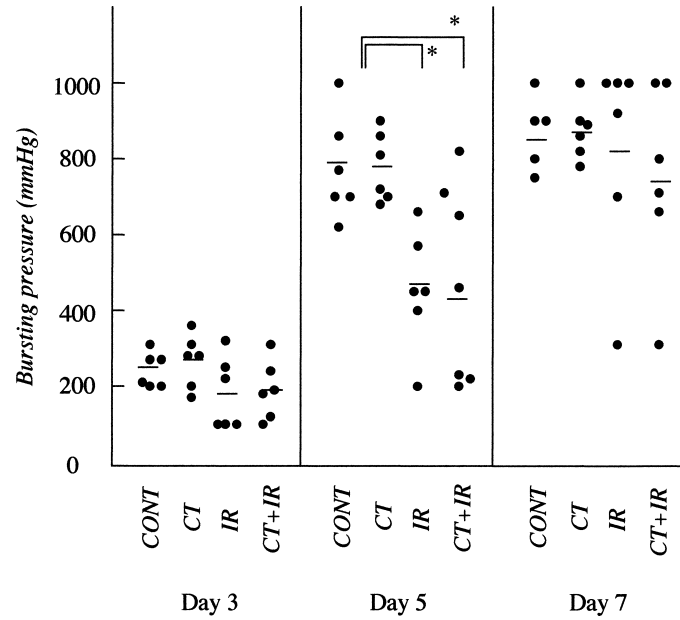


Fig. 8. Anastomotic bursting pressures in experiment 2.

Each point presents a single anastomosis. The horizontal lines represent the mean values.

\*The mean values of IR and CT+IR groups are significantly lower than those of the control and CT groups ( $p < 0.05$ ). For abbreviations, see Fig. 2.

TABLE 2. *Anastomotic hydroxyproline contents in experiment 2*

Group	Day 3	Day 5	Day 7
CONT	149.8±8.4	168.2±18.3	201.3±31.0
CT	151.0±25.3	180.3±34.6	204.7±23.5
IR	200.1±56.9*	253.3±40.4*	200.8±47.8
CT+IR	213.0±13.6**	265.7±28.7**	214.1±51.1

Anastomotic hydroxyproline contents are expressed mean (n mol/mg dry weight)±S.D. \* $p < 0.01$ , significantly higher than in CONT group. \*\* $p < 0.01$ , significantly higher than in CONT group and CT group. For abbreviations, see Fig. 2.

the bursting pressures were significantly lower in IR ( $463 \pm 157$  mmHg) and CT+IR ( $425 \pm 262$  mmHg) groups compared with the control ( $797 \pm 146$  mmHg) and CT ( $782 \pm 96$  mmHg) groups ( $p < 0.05$ ). At postoperative day 7, there were no significant differences in bursting pressure among the groups (Fig. 8).

*Hydroxyproline content.* At postoperative days 3 and 5, the hydroxyproline content at the site of anastomosis was significantly higher in

IR group than in the control group ( $p < 0.01$ ). At postoperative days 3 and 5, the content was significantly higher in CT+IR group than in the control and CT groups ( $p < 0.01$ , Table 2). At postoperative day 7, there were no significant differences in hydroxyproline contents among the four groups.

*Histopathological findings.* No signs of submucosal connective tissue regeneration were noted, and defects were seen in the submucosal tissue in IR and CT+IR groups compared with the findings in the control and CT groups at postoperative days 3 and 5 (data not shown). Mature connective tissue was identified in IR and CT+IR groups at postoperative day 7 compared with the control group.

## DISCUSSION

The long-term survival rate of patients with stage III NSCLC is poor due to the frequent spread of micrometastasis. The median survival time following surgical resection in patients with clinically evident mediastinal lymph node metastasis is 12 month, with only 10



to 20% of patients surviving 3 years (Martini et al. 1980; Pearson et al. 1982; Cybulsky et al. 1992). Attempts to prolong survival following surgical resection by induction chemotherapy or by a combination of chemotherapy and radiotherapy have improved the median survival time and rate in randomized clinical trials (Dillman et al. 1990; Le Chevalier et al. 1991; Rosell et al. 1994). However, adverse effects of acute irradiation on healing of the bronchial anastomosis have been documented both experimentally and clinically (Tsubota et al. 1975; Inui et al. 1993). Although preoperative chemotherapy combined with radiotherapy appears to influence postoperative tracheobronchial wound healing, few studies have examined such effects in detail. Therefore, our study examined the effects of preoperative irradiation and anti-neoplastic agents on bronchial anastomotic healing in rats.

We recently showed that preoperative thoracic irradiation clearly impaired bronchial anastomotic healing in rats treated with a single dose of 30 Gy and 40 Gy at day 7 before surgery (Masao 2001). Several adverse effects on healing were noted when the radiation dose exceeded 30 or 40 Gy. In our experimental study, there were significant differences in the bursting pressure and histopathological findings among rats that received an IR dose of 30 or 40 Gy compared with 20 Gy at day 7 after operation. Since we wanted to examine the synergistic adverse effects of PE on healing of bronchial anastomosis, when simultaneously administered at the time of irradiation, the experiment was designed therefore to use a dose of only 20 Gy in this study. According to the linear-quadratic concept and assuming an  $\alpha/\beta$  ratio of 20 Gy for acute effects, the extrapolated tolerance dose of 25 Gy and 50 Gy are approximately the biological equivalent for acute effects with a single dose of 20 Gy and 30 Gy, respectively (Fowler 1989; Tsubiana 1990). Clinical and experimental data have suggested that bronchial complications seem to increase when preoperative radia-

tion dose exceeds 35 to 40 Gy (Tsubota et al. 1975; Inui et al. 1993).

Preoperative thoracic irradiation and simultaneous administration of PE have shown acceptable toxicity and relatively good survival results (Yashar et al. 1992; Bonomi 1993). Several studies used cisplatin as a radiosensitizer in combination with radiotherapy (Ruckdeschel et al. 1986; Bunn 1989). However, our results showed no significant differences in the bursting pressure between the irradiation group and radiochemotherapy group. Thus, PE combined with irradiation does not seem to have a synergistic adverse effect on wound healing of bronchial anastomosis in rats. On the other hand, our results showed a significant difference in the bursting pressure between the irradiation and chemotherapy groups, indicating that thoracic irradiation produces more serious adverse effects on wound healing (compared to chemotherapy), at least in our rat model.

Clinically, significant leukopenia was noted at 2 or 3 weeks after chemotherapy, and recovered gradually, and were already improved at 3 or 4 weeks. But, in our rat model, significant leukopenia was noted in CT and CT+IR groups at day 6 after chemotherapy and recovered gradually, and were already improved at day 10 after chemotherapy. The platelet counts showed same change as leukocyte counts. We expect that regeneration speed in bone marrows of rats was equal to double that of human.

In our study, the bursting pressure in irradiation and radiochemotherapy groups was significantly lower than the control and chemotherapy group at day 5 ( $p < 0.05$ , each). Histopathological examination of the site of anastomosis using H & E stained sections showed lack of submucosal connective tissue regeneration and defects in the submucosal tissue in the irradiation group and radiochemotherapy group at postoperative days 3 and 5. However, the healing process was not delayed in the latter two groups compared with the control at pos-

operative day 7. The cytotoxic effects of chemotherapy and irradiation include among others, the prevention of fibroblast proliferation, collagen synthesis, and deposition of collagen on the anastomotic line, which adversely affect wound healing and anastomotic breaking strength (Kuzu et al. 1999). Previous studies reported that depletion of macrophages is one of the most important causes of impaired wound healing in chemotherapy, in addition to other mechanisms (Shirafuji et al. 2001). T lymphocytes commence to accumulate in the wound area at 5 days after injury (Barendsen 1982). In our study, depletion of leukocytes and macrophages was not seen in irradiation group, compared with the control group. It is possible that impairment of the healing process could be due to another important factor. The healing of bronchial anastomosis is closely related to regional mucosal blood flow. Preoperative irradiation decreases bronchial mucosal blood flow and adversely affects the healing of bronchial anastomosis. In the early post-irradiation period, tissue shows reactive congestion, edema, and inhibition of capillary proliferation. These changes may cause a reduction in bronchial mucosal blood flow particularly during the acute period. However, recovery of bronchial mucosal blood flow seems to occur within 8 to 10 days in the human bronchus (Yamamoto et al. 2000). Our study suggested recovery of the healing process in bronchial anastomosis within 7 days based on measurements of the bursting pressure and histopathological findings. These results emphasize the importance of careful management of the healing of anastomotic area in the early stages (the first five postoperative days).

Hydroxyproline makes up to 10% of collagen, and since it is found only sparingly in other proteins, it is used as an indirect marker for collagen contents (Hoshi et al. 1993; Dominguez et al. 1996). Our results showed that hydroxyproline contents increased in irradiated rats, although this result did not

parallel the bursting pressure. Several studies reported significantly high hydroxyproline levels in irradiated group than the control (Jahnsen et al. 1995; Dominguez et al. 1996). Whether the collagen in the irradiated tissues differs in type or structure from that of non-irradiated bowel, the exact reason for the higher hydroxyproline concentrations is unknown at present. Collagen concentrations per unit weight should be considered with caution since they may change as a consequence of changes in non-collagenous substances. Since no distinct correlation have been demonstrated between development of mechanical strength and collagen levels in the healing anastomosis, attention should not be restricted to a description of the quality of collagen present (Hendriks and Mastboom 1990). Further studies examining collagen maturation and structural organization are needed.

In conclusion, we have demonstrated in the present study that the healing of bronchial anastomosis was markedly delayed in early postoperative days in rats that had received radiochemotherapy. There was a significant difference in wound healing process at postoperative day 5 in radiochemotherapy group, suggesting that further exhaustive evaluation of this therapeutic regimen, particularly that of irradiation, is necessary.

### Acknowledgments

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