Social Isolation Stress Impairs the Resistance of Mice to Experimental Liver Metastasis of Murine Colon 26-L5 Carcinoma Cells

Wenjuan Wu, Jun Murata, Kazuko Hayashi, Takeshi Yamaura, Noriyasu Mitani, and Ikuo Saiki*

Department of Pathogenic Biochemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–0194, Japan. Received January 5, 2001; accepted March 26, 2001

Our previous study has demonstrated that the exposure of male BALB/c mice to social isolation stress caused a suppressed immune response and enhanced liver metastasis of colon 26-L5 carcinoma cells. To more precisely understand the influence of psychosocial factors on the metastatic process, here we have investigated the effect of social isolation stress on the vulnerability of the host to develop liver metastasis of colon 26-L5 cells, including the time span and incidence of metastatic formation, survival time and chemotherapy response. Isolation stress decreased the time period required for the metastasis formation relative to that in controls. On day 7 after the tumor injection, the 75% incidence of tumor metastasis in the stressed mice was 5 times the 15% incidence in the unstressed mice. When exposed to the challenge of lower cell numbers (0.025, 0.05, 0.1×10⁷/mouse) of colon 26-L5 cells, mice subjected to isolation stress developed an elevated incidence of metastasis (33.3, 66.6, and 100%, respectively) as compared with the controls (0, 33.3 and 50%, respectively). The survival time following the tumor inoculation was also shorter in the stressed mice (21.83±1.59 d) than in the control mice (24.08±1.68 d). Furthermore, the response of liver metastasis to chemotherapy consisting of 2 mg/kg cisplatin (CDDP) was worse in the stressed mice than that in unstressed mice. These findings suggested that social isolation stress could significantly impair the resistance of mice to the development of metastasis.

Key words social isolation; stress; metastasis; chemotherapy; cancer survival; colon cancer

Recently, growing epidemiological evidence suggests the importance of psychosocial factors in a wide variety of diseases such as depression, cardiovascular diseases and cancer.¹—³ Social isolation, divorce and bereavement are reported to be associated with an increased risk of cancer recurrence, the probability of metastasis and mortality rates for cancer, as well as decreased efficacy of cancer therapy.⁴—⁷ In contrast, reducing the impact of psychosocial stress through social support, including the presence of a social network or psychosocial intervention, has also been shown to be related to an increase in the survival time and a decrease in the rate of metastasis.⁸,⁹

Experimental investigations into the effects of psychosocial factors on tumor progression in animals may provide useful information of basic values for clinical situations. Social isolation (individual housing) is a model of lack of social interactions among animals, which is relatively comparable with the situation of humans who feel isolated. In rodents, social isolation results in marked behavioral disturbances such as increased aggressiveness, enhanced locomotor activity and reduced pentobarbital-induced sleeping time,¹⁰,¹¹ and physiological disturbances, including high levels of plasma corticosterone, catecholamine and high activity of corticotropin releasing factor (CRF).¹²—¼ Because of its inherent social nature, social isolation is viewed as a natural and convenient model of psychosocial stress, and would be helpful for investigating the modulatory role of psychosocial stress in tumor development. Social isolation stress has been reported to accelerate the development and growth of either transplanted or chemically induced tumors, and to attenuate the response of tumors to chemotherapy.¹⁵—¹⁷ However, there are also some conflicting reports on the association between tumor development and psychosocial stress in both human and animal studies, because of the variations in stress chronicity, timing of stress and types of tumors tested, etc.¹⁸,¹⁹ Nevertheless, the strong evidence indicates that the tumor progression can be affected by psychosocial factors relating to grouping and isolation, although the underlying mechanism is still unknown.

Despite the advances in diagnostic techniques and therapeutic modalities for malignant tumors including colon cancer, the mortality rate of cancer is still high.²⁰ Many cancer patients develop recurrent and metastatic cancer even if curative surgery is undergone. Metastasis is a major cause of mortality in cancer. However, to our knowledge, the specific effects of psychosocial stress on tumor recurrence and metastasis have only been marginally studied, in spite of the crucial clinical relevance of these phenomena.

We recently found that social isolation stress significantly suppressed the basal cellular immune responses and exacerbated experimental liver metastasis of colon 26-L5 carcinoma cells in male BALB/c mice.²¹ The immune surveillance functions of effector cells such as T and B lymphocytes, NK cells and macrophages are considered to play an important role in controlling tumor metastatic processes.²²,²³ Thus, the impairment of immune functions by isolation stress may theoretically affect the ability of the host to respond adequately to metastasis formation and influence the biological behavior of metastasis.

To further clarify the effect of psychosocial stress on cancer metastasis, we focused here on investigating the influence of social isolation stress on the vulnerability of the host to develop liver metastasis of colon 26-L5 carcinoma cells in terms of time span and incidence of metastasis formation, the extent of metastatic tumor burden, chemotherapy response and survival time.

MATERIALS AND METHODS

Chemical Cisplatin (cis-diamine dichloro platinum II, CDDP) was kindly provided by Nippon Kayaku Co., Ltd.

Animals Four-week-old BALB/c male mice weighing
16—19 g were obtained from Japan SLC, Inc. (Hamamatsu). After they were acclimated to the laboratory for 3 or 4 d in group-housing conditions, they were randomly assigned to be group-housed \((n=4—6\) per cage: \(35\times 30\times 18\) cm) or individually housed \((n=1\) per cage: \(24\times 17\times 12\) cm) in an animal laboratory which was maintained at constant temperature \((23—25^\circ \text{C})\) and relative humidity \((65%)\) with a 12 h light/dark cycle. There was a wall and a 12 cm distance between two cages for the individual housing. Food and water were available \textit{ad libitum}. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Cell Line** The liver metastatic tumor cell line of colon 26 carcinoma (colon 26-L5) was obtained by the \textit{in vivo} selection method.\(^24\) Colon 26-L5 cells were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and l-glutamine at 37°C in a humidified atmosphere of 5% \textit{CO}_2/95% air.

**Experimental Liver Metastasis of Colon 26-L5 Cells**
Log-phase cultures of colon 26-L5 cells were harvested with 1 mM EDTA in phosphate buffered saline (PBS), washed three times with serum-free RPMI-1640 medium and resuspended at appropriate concentrations in RPMI-1640. Colon 26-L5 cells \((0.025, 0.05, 0.1, 1.0\) or \(2.0\times 10^5/200 \mu\text{L/mouse})\) were implanted in the mice under ether anesthesia \textit{via} intraportal vein injection, as described previously.\(^25\) The mice were sacrificed on day 7, 10, 13 (or 14) or 17 following tumor inoculation and the livers were removed. The increase in liver weight and the number of tumor colonies in the liver were measured to evaluate tumor metastasis.

**Histopathological Analysis of Metastatic Liver**
Mice inoculated with colon 26-L5 cells as described above were killed on day 7, 10 or 13 after the inoculation. Hematoxylin-eosin staining was performed using 4\(\mu\)m paraffin-embedded tumor-bearing liver sections. Specimens were de-paraffinized with xylene, immersed in graded ethanol and stained with hematoxylin for 1 min, then with eosin for 4 min.

**Survival Probability in Colon 26-L5-Bearing Mice**
Colon 26-L5 \((1\times 10^4)\) cells were intraportally inoculated into mice \((n=12)\) that had been group-housed or socially isolated for 14 d, and the same housing conditions were continued throughout the experiment. The survival probability was determined as the period when the tumor-bearing mice lived until they succumbed to the tumor burden.

**Response of Mice to Chemotherapy**
The experimental liver metastasis model was used as described above. CDDP \((2, 4\, \text{mg/kg})\) or vehicle (saline) was administered once i.v. on day 3 after the injection of \(1\times 10^4\) tumor cells. The mice were sacrificed on day 14 following tumor implantation. The increase in liver weight and the number of tumor colonies in the liver were measured, and the difference between the drug-treated mice and vehicle-treated mice was evaluated as the chemotherapy response to CDDP of the mice.

**Statistical Analysis** The differences between the groups were determined by Student’s two-tailed \(t\)-test or the Mann–Whitney \(U\) test. A \(p\)-value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Effect of Social Isolation Stress on the Time Period for Liver Metastasis Formation of Colon 26-L5 Cells**
We first investigated the influence of isolation stress on the period required for liver metastasis formation caused by the intraportal vein injection of colon 26-L5 carcinoma cells in syngeneic male BALB/c mice. Mice were individually housed or group-housed for 2 weeks before tumor inoculation, and remained thus housed throughout the experiment. The metastatic tumor burden, expressed as the increase in liver weight and colony number, was measured on day 7, 10, 13 or 17 after tumor inoculation. As shown in Figs. 1 and 2, 24), the period until metastasis formation was markedly influenced by isolation stress. Earlier manifestation of metastatic colonies was found in the stressed group relative to the group-housed control. For example, on day 7 following tumor injection, no liver metastasis was macroscopically observed in 75% \((3/4)\) of the group-housed mice, while 4—6 tumor colonies per liver were histopathologically detected in the 75% \((3/4)\) of the socially isolated mice. In accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Fig. 1. Time Course of Liver Metastasis of Colon 26-L5 Carcinoma Cells in Group-Housed or Socially Isolated Mice**
Male BALB/c mice \((n=4\) or 5) were group-housed or individually housed for 2 weeks before intraportal \textit{vein} \((i.p.v.)\) injection of colon 26-L5 cells \((1\times 10^4)\) and throughout the experiment. The mice were sacrificed on day 7, 10, 13 or 17 after tumor inoculation, and the increase in liver weight and number of tumor colonies in the liver were measured to evaluate liver metastasis. Each value represents mean \(\pm\) S.D. of 4—5 mice. *: \(p<0.05\), **: \(p<0.01\), vs. group-housed control, by Student’s two-tailed \(t\)-test.

**Fig. 2. Histopathological Analysis of Metastatic Liver**
Mice inoculated \textit{i.p.v.} with colon 26-L5 cells \((1\times 10^4)\) were killed on day 7, 10 or 13 after the inoculation. Representative liver sections from the group-housed (A, C, E) and isolated mice (B, D, F) were prepared for hematoxylin-eosin staining. T, tumor tissue; L, liver tissue. Magnification: \(\times 100\).
with the above finding, the tumor burden markedly increased as a function of stress throughout the experiment, as compared with the controls.

Effect of Social Isolation Stress on the Liver Metastasis Formation Produced by Various Cell Numbers of Tumor Cells

To examine the effect of isolation stress on the susceptibility to liver metastasis formation, different cell numbers (2.5×10^2 to 2.0×10^4/mouse) of colony 26-L5 cells were injected intraportally into the group-housed or socially isolated mice. As shown in Table 1, no visible metastatic colonies were detected in the liver of group-housed mice inoculated with 2.5×10^2 tumor cells, while 3—6 tumor colonies in the liver were observed in 33% (2/6) of the socially isolated mice. Similarly, an elevated incidence of metastasis formation was found in the socially isolated mice inoculated with 5.0×10^2 or 1.0×10^3 tumor cells, as compared with the group-housed mice. When 1.0×10^3 or 2.0×10^4 tumor cells were injected into the mice, the incidence of liver metastasis was observed in all mice, regardless of the stress, but the number of metastatic colonies in the liver was significantly increased in the stressed mice as compared with the unstressed mice.

Effect of Social Isolation Stress on the Survival Rate of the Group-Housed or Socially Isolated Mice Bearing Liver Metastasis

The influence of social isolation stress on the survival probability of the mice bearing liver metastasis is shown in Fig. 3. Social isolation stress resulted in a remarkable enhancement of mortality, probably due to the increase of liver metastasis. Decreased mean survival time was much more pronounced in the socially isolated mice than in the controls.

Effect of Social Isolation Stress on the Chemotherapy Responses of the Group-Housed or Socially Isolated Mice Bearing Liver Metastasis

To further confirm that isolation stress could affect the resistance of the host to developing metastasis, we evaluated the chemotherapy response of the metastatic tumor in the group-housed or socially isolated mice. CDDP was administered i.v. to the mice on day 3 after the tumor inoculation. As shown in Figs. 4A and B, the administration of 2 mg/kg CDDP significantly suppressed the liver metastasis (expressed as the increase in liver weight and colony number) only in group-housed mice (being significantly inhibited from 0.929±0.278 g and 79.3±10.8 to 0.421±0.219 g and 55.5±13.4, respectively), and not in socially isolated mice (being inhibited from 1.888±0.459 g and 144.5±43.4 to 1.397±1.424 g and 121.8±31.4, respectively), while 4 mg/kg CDDP obviously inhibited the tumor burden in both groups. The results in Figs. 4C and D were expressed as the percent inhibition of the increase in liver weight and the percent inhibition of tumor colony number. The stressed mice showed a tendency to have a poorer response to the treatment with 2 mg/kg CDDP than the unstressed mice, although the difference between them was not significant (p=0.07, Fig. 4C).

**Table 1. Experimental Liver Metastasis of Colon 26-L5 Carcinoma Cells in Group-Housed or Socially Isolated Mice**

<table>
<thead>
<tr>
<th>No. of cells inoculated</th>
<th>Group</th>
<th>Incidence (%)</th>
<th>Increase of liver weight (g±S.D. (range))</th>
<th>No. of tumor colonies in liver mean±S.D. (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5×10^2</td>
<td>Group-housing</td>
<td>0/5 (0)</td>
<td>0.283±0.094 (0.146—0.369)</td>
<td>1.300±2.443 (0—6)</td>
</tr>
<tr>
<td></td>
<td>Social isolation</td>
<td>2/6 (33)</td>
<td>0.448±0.006 (0.393—0.548)**</td>
<td>0.333±0.516 (0—1)</td>
</tr>
<tr>
<td>5.0×10^2</td>
<td>Group-housing</td>
<td>2/6 (33)</td>
<td>0.168±0.136 (0.004—0.290)</td>
<td>0.750±0.957 (0—2)</td>
</tr>
<tr>
<td></td>
<td>Social isolation</td>
<td>4/6 (67)</td>
<td>0.218±0.108 (0.046—0.342)</td>
<td>9.257±5.377 (3—16)**</td>
</tr>
<tr>
<td>1.0×10^3</td>
<td>Group-housing</td>
<td>2/4 (50)</td>
<td>0.218±0.032 (0.180—0.257)</td>
<td>0.108±0.342 (0—3)</td>
</tr>
<tr>
<td></td>
<td>Social isolation</td>
<td>4/4 (100)</td>
<td>0.301±0.096 (0.227—0.437)</td>
<td>34.750±13.020 (23—47)*</td>
</tr>
<tr>
<td>1.0×10^4</td>
<td>Group-housing</td>
<td>4/4 (100)</td>
<td>0.523±0.279 (0.323—0.800)</td>
<td>70.000±25.430 (39—97)**</td>
</tr>
<tr>
<td></td>
<td>Social isolation</td>
<td>4/4 (100)</td>
<td>0.866±0.459 (0.397—1.424)</td>
<td>76.500±16.280 (57—95)</td>
</tr>
<tr>
<td>2.0×10^4</td>
<td>Group-housing</td>
<td>4/4 (100)</td>
<td>1.586±0.876 (0.857—2.819)</td>
<td>153.500±31.860 (129—200)**</td>
</tr>
<tr>
<td></td>
<td>Social isolation</td>
<td>4/4 (100)</td>
<td>3.609±1.024 (3.056—5.144)**</td>
<td></td>
</tr>
</tbody>
</table>

Male BALB/c mice per group (n=4—6) were individually housed for 2 weeks before i.p.v. injection of colon 26-L5 cells (0.025, 0.05, 0.10, 1.0 and 2.0×10^3, respectively) and throughout the experiment. After the mice were sacrificed on day 14 after tumor inoculation, the incidence, increase in liver weight and the number of tumor colonies in the liver were measured to evaluate liver metastasis. *: p<0.05; **: p<0.01, vs group-housed control, according to the Student’s two tailed t-test.

DISCUSSION

Our recent study demonstrated that social isolation stress enhanced the liver metastasis of colon 26-L5 carcinoma cells in terms of increases in liver weight and tumor colony number in male BALB/C mice. The present study supported...
and extended these previous findings, demonstrating that social isolation stress impaired the resistance of mice against metastatic formation by accelerating the manifestation of tumor colonies, increasing the incidence of metastasis and enhancing mortality, as well as reducing the chemotherapy response (Table 1, Figs. 1 — 4).

Mounting evidence in the literature about the relationship between stress and cancer provides increasing support for an association between psychosocial factors and tumor progression. People with few social connections show significantly poorer cancer survival rates. Those who had more stressful life events were at increased risk for the recurrence of cancer and at even higher risk for death from cancer. Loss of a spouse is related to an increase in the mortality rate for cancer. Moreover, in patients with metastatic non-small cell lung cancer and breast cancer, the combination of supportive care and chemotherapy offers a survival advantage over either modality alone. The available studies have established the idea that a helpless response to psychosocial stress may actually have a stronger influence on tumor progression.

Considering the metastatic process, it is possible that the isolation stress influences a variety of steps during tumor metastasis, including the direct stimulation of tumor growth at metastatic sites and the stimulation of angiogenesis through the activity of the hypothalamus-pituitary adrenocortical (HPA) axis, as well as through the suppression of cellular immunity, facilitating tumor metastasis. Furthermore, the modification of behaviors in mice under isolation stress must also be considered as a possible link between the stress and metastasis development. For example, the strong anxiety and sleep disturbance induced by isolation stress have been suggested to impair resistance to disease progression. Consequently, the behavioral and physiological disturbances resulting from the isolation stress may be significantly involved in suppressing resistance to tumor cells and in promoting the subsequent metastasis. However, the exact biological mechanisms underlying the effect of isolation stress on liver metastasis still needs to be investigated.

In general, chemotherapy has been shown to be more effective against fast-growing tumors. The inhibition of liver metastasis by treatment with 2 mg/kg CDDP in the group-

---

**Fig. 4.** Chemotherapy Response to CDDP of Group-Housed or Socially Isolated Mice Bearing Liver Metastasis of Colon 26-L5 Carcinoma Cells

Male BALB/c mice (n = 5 or 6) were group-housed or individually housed for 2 weeks before i.p. injection of colon 26-L5 cells (1 x 10^6) and then throughout the experiment. CDDP (2 or 4 mg/kg) was administered i.v. to the mice on day 3 after tumor inoculation. After the mice were killed on day 14 following the implantation of tumor cells, the liver metastasis was expressed as the increase in liver weight (A) and colony number (B). Percent Inhibition of increase of liver weight (C) and colony number (D) was expressed as the percent inhibition of control (saline) in the same groups, respectively. Each value represents mean ± S.D. of 5—6 mice. *: p < 0.05, **: p < 0.01, ***: p < 0.001, vs. group-housed control, according to the Student’s tailed t-test.
housed mice was greater than that in the isolated mice. This result indicates that metastasis in the stressed mice responded worse to chemotherapy than did metastasis in the unstressed mice. It is possible that the tumor growth rate is so rapid or the metastasis formation is so early in mice under isolation stress that the tumor burden becomes resistant to the therapy with 2 mg/kg CDDP (but not 4 mg/kg). Also, a poorer response to chemotherapy in the stressed mice may be associated with an alteration of drug metabolism. The shorter survival rate in the socially isolated mice may also result from the metastasis developing early due to isolation stress. This further supports the hypothesis that social isolation stress suppresses the resistance of the host toward developing metastasis.

In summary, our data suggested that isolation stress results in decreased resistance of the host to developing metastasis. These data highlight the possible impact of psychosocial stress on the complex interrelationships among the host environment, tumor metastasis and the efficacy of chemotherapy, and seem to have interesting experimental and clinical implications. Further investigation of the mechanism underlying the effects of isolation stress on metastasis is warranted, and is now in progress, aiming to identify and characterize the neuroendocrine and immunological mediators acting in response to the stress.

REFERENCES