Triple Paraneoplastic Syndrome of Hypercalcemia, Leukocytosis and Cachexia in Two Human Tumor Xerografts in Nude Mice

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Nude mice bearing the human oral cavity carcinoma cell line OCC-1, and the lung cancer cell line LC-1, developed a triple paraneoplastic syndrome consisting of hypercalcemia, cachexia and leukocytosis. All of these abnormalities disappeared rapidly after surgical resection of the tumors, suggesting their ectopic humoral nature. Search for the factors responsible for the respective abnormalities revealed that the production of parathyroid hormone-related protein and colony-stimulating factors (CSFs), mainly granulocyte-CSF, by the tumors could explain the hypercalcemia and leukocytosis, respectively. With regard to the severe cachexia, the production of two cachexia-associated cytokines, interleukin-6 and leukemia inhibitory factor, was able to explain the syndrome in OCC-1-bearing nude mice; however, the factor responsible in LC-1-bearing nude mice could not be identified. The triple paraneoplastic syndrome that developed in these two animal models could be explained partly by concomitant production of the peptide hormone and cytokines by cancer cells. These animal models may be very useful for the evaluation of diagnostic and therapeutic modalities for humoral abnormalities.


Key words: Hypercalcemia—Cachexia—Leukocytosis—Parathyroid hormone-related protein—Cytokines

Introduction

Improvement of the quality of life (QOL) in cancer patients is becoming an important issue in modern medical oncology. Paraneoplastic syndromes are the major cause of poor QOL, and ectopic humoral abnormalities play a substantial role in their development. Multiple paraneoplastic syndromes involving two or more abnormalities are rare clinical entities in cancer patients with advanced disease, and unique animal models of these syndromes may be useful for further understanding of these morbidities.

In this study we analyzed two animal models of a triple paraneoplastic syndrome consisting of hypercalcemia, leukocytosis and cachexia, anticipating that clarification of the etiology of this syndrome might be helpful for developing new therapeutic modalities for cancer patients with similar syndromes.

Materials and Methods

Cancer Cells

The OCC-1 tumor cell line was established from a squamous cell oral cavity carcinoma obtained from a patient manifesting hypercalcemia and leukocytosis. The LC-1 tumor cell line was established from a squamous cell lung carcinoma obtained from a patient with neutrophilia. These tumors were kindly provided by the Central Institute for Experimental Animals (Kawasaki, Kanagawa) and maintained in our laboratory by successive subcutaneous transplantation into nude mice.

Animal Experiments

Seven- to eight-week old female BALB/c-nu/nu nude mice weighing 20 to 25g were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka). They
Relationship between Tumor Volume and Development of Hypercalcemia, Leukocytosis and Cachexia

A total of 45 nude mice were used for the OCC-1 experiment to study hypercalcemia and leukocytosis. These nude mice were divided into groups of control and tumor-bearing mice with tumor volumes of approximately 50, 100, 200, 400, 600, 800 and 1000 mm$^3$. Blood samples were first obtained from the orbital sinus of the nude mice for determination of the plasma calcium level, and then blood was withdrawn from the abdominal vena cava into heparinized syringes for determination of other parameters. The spleen index was then calculated as follows:

\[
\text{spleen index} = \frac{\text{spleen weight (g)}}{\text{carcass body weight (g)}} \times 100
\]

Here, the carcass body weight was calculated as:

\[
\text{whole body weight (g)} - \frac{\text{tumor volume (mm}^3\text{)}}{1000}
\]

In the study of leukocytosis syndrome, five control nude mice and 10 OCC-1-bearing nude mice were examined. When the tumor volume reached approximately 900 mm$^3$, blood samples were obtained from the abdominal vena cava into heparinized syringes for determination of the white blood cell (WBC) count and plasma granulocyte colony-stimulating factor (G-CSF) level.

In the LC-1 experiment, a total of 41 nude mice were used to study hypercalcemia, cachexia and leukocytosis. They were divided into groups of control and tumor-bearing mice with tumor volumes of approximately 200, 400, 600, 700, 1000, 2000, 3000 and 4000 mm$^3$. Blood samples were obtained and the spleen index was calculated in the same way as for the OCC-1-bearing nude mice.

Tumor Resection

This experiment was performed to determine whether factors produced by the transplanted tumors caused the hypercalcemia, leukocytosis and cachexia. Five nude mice were used as controls and 10 nude mice bearing OCC-1 tumors and nine bearing LC-1 tumors were analyzed. After tumor transplantation, carcass body weight was calculated twice or three times per week. After the blood had been obtained, the tumors were surgically resected from five nude mice when the carcass body weight had decreased to 80% that of control nude mice. Plasma was obtained from the orbital sinus of nude mice at three points. In the case of OCC-1, these procedures were done before tumor resection and on the second and fourth days after resection, and in the case of LC-1, before, and on the second and fifth days after resection. In the OCC-1 experiment, the levels of calcium and immunosuppressive acidic protein (IAP) in plasma were determined in each nude mouse, and in the LC-1 experiment, the plasma levels of calcium, parathyroid hormone-related protein (PTHrP) and G-CSF were determined. For determination of the plasma PTHrP and G-CSF levels, pooled plasma for each group was used.

Biochemical Studies

The WBC count and plasma calcium level were determined immediately with an auto-hemalyzer Celltac MEK-5158 (Nihon Kohden Corp., Tokyo) and with an auto-biochemical analyzer (FUJI DRY-CHEM 5500; Fuji Medical System Co., Ltd., Tokyo) using chlorophosphonazo-III, respectively. The remaining plasma samples were stored at -20°C until measurement of other parameters. The plasma PTHrP level was determined by a human PTHrP radiomunnoassay (Dai-ichi Radioisotope Laboratories, Ltd., Tokyo), and the plasma interleukin (IL)-6, leukemia inhibitory factor (LIF) and G-CSF levels were determined with human IL-6, human LIF and human G-CSF immunoassay kits (R&D Systems, Minneapolis, MN), respectively. The plasma IAP level was determined using mouse IAP plates (Sanko Jun-yaku Co., Tokyo).

Northern Blot Analyses

Tumor tissues with volumes of approximately 1000 and 2000 mm$^3$ were obtained from five OCC-1 and LC-1-bearing nude mice, respectively, showing the triple paraneoplastic syndrome. The tissues were homogenized in liquid nitrogen, and then total cellular RNA was extracted from the homogenate by the acid guanidinium-phenol-chloroform method. After selection of poly(A)$^+$ RNA by Oligotex dT-30 affinity chromatography, each sample (5 μg per lane) was electrophoresed and Northern blot hybridization was performed as described previously. Synthetic oligodeoxyribonucleotides (39–60 mer) were used for detecting mRNA of each gene, except for the IL-6 gene. The synthetic probes were complementary to the following portions of the mRNAs: the coding sequence corresponding to nucleotides 292–342 of human PTHrP, 292–345 of human LIF;
Fig. 1. Relationship between tumor volume and plasma calcium and PTHrP levels in OCC-1-bearing nude mice (a) and LC-1-bearing nude mice (b). Data represent mean ± SD of three or five nude mice. Significant increase from the control levels is expressed as * and ** for \( P < 0.05 \) and \( P < 0.01 \), respectively. PTHrP, parathyroid hormone-related protein.

91–150 of human G-CSF\(^{13}\); 97–135 of human macrophage (M)-CSF\(^{13}\); 1–57 of human granulocyte-macrophage (GM)-CSF.\(^{13}\) A 535-base human IL-6 complementary DNA probe corresponding to nucleotides 174–708 in the coding region was also used. The expression of \( \beta \)-actin mRNA was examined to determine the integrity of poly(A)+ RNA samples.

Statistics

Statistical analysis was performed by Student’s \( t \) test.

Results

Tumor Growth

Several days after transplantation, “take” of the OCC-1 tumors in the nude mice was observed, when the volume was approximately 50 mm\(^3\). Subsequently, the tumors grew rapidly, the volume doubling every two days, and all OCC-1-bearing nude mice died within 3 weeks after transplantation.

“Take” of the LC-1 tumors in nude mice was seen about 2 weeks after transplantation, when the tumor volume was approximately 100 mm\(^3\). Within one month, the tumor volume reached 1000 mm\(^3\), and all LC-1-bearing nude mice died within 2 months after transplantation.

Hypercalcemia

Fig. 1 shows the relationship between tumor volume and the plasma levels of calcium and PTHrP in tumor-bearing nude mice. In OCC-1-bearing mice (Fig. 1, left panel), a slight but significant increase in the plasma PTHrP level was observed when the tumor volume reached 50 mm\(^3\), and thereafter the plasma calcium level began to increase when the tumor volume reached 200 mm\(^3\). In LC-1-bearing nude mice (Fig. 1, right panel), the plasma PTHrP level increased when the tumor volume reached 400 mm\(^3\), followed by a significant increase in the plasma calcium level when the tumor volume reached 600 mm\(^3\). It is noteworthy that the increase in the plasma PTHrP level preceded that in the plasma calcium level in both animal models.

Leukocytosis

Fig. 2 shows the relationship between tumor volume, plasma G-CSF level, WBC count and
TRIPLE PARANEOPlastic SYNDROME

Fig. 3. Relationship between tumor volume and carcass body weight (a), plasma IAP level (b), plasma IL-6 level (c) and plasma LIF level (d) in OCC-1-bearing nude mice. Data represent means ± SD of five nude mice. In columns (a) and (b), the significant difference from the control levels is expressed as * and ** for \( P<0.05 \) and \( P<0.01 \), respectively. In the columns (c) and (d), broken lines indicate the limits of detectability (IL-6, 16 pg/ml; LIF, 5 pg/ml). IAP, immunosuppressive acidic protein; IL, interleukin; LIF, leukemia inhibitory factor.

The carcass body weight decreased markedly with tumor growth; it was decreased significantly in nude mice bearing tumors larger than 100 mm\(^3\). The plasma IAP, IL-6 and LIF levels were increased in the tumor-bearing mice, and higher levels were observed in mice bearing larger tumors.

In the OCC-1-bearing nude mice, the WBC count, plasma G-CSF level and spleen index were determined at one point, two weeks after transplantation (data not shown). OCC-1-bearing nude mice had leukocytosis with a WBC count ranging from 12,000 to 28,000/\( \mu l \), 3.2- to 7.7-fold higher than that of control nude mice. The mean plasma G-CSF level was 3800-fold higher than the minimum detectable concentration. The spleen index was approximately 3.7 times that in the control nude mice, and the spleen contained a large number of neutrophils and megakaryocytes.

Cachexia

Fig. 3 shows the relationship between tumor volume, carcass body weight and plasma levels of IAP, IL-6 and LIF in OCC-1-bearing nude mice. The carcass body weight decreased markedly with tumor growth; it was decreased significantly in nude mice bearing tumors larger than 100 mm\(^3\). The plasma IAP, IL-6 and LIF levels were increased in the tumor-bearing mice, and higher levels were observed in mice bearing larger tumors.

In the LC-1-bearing nude mice, the carcass body weight change in OCC-1-bearing nude mice (a) and LC-1-bearing nude mice (b). Each point represents mean carcass body weight of four or five nude mice. Since the tumor growth of LC-1 was not very rapid, the mean relative carcass body weight on day 35 is taken as 1.0. The significant difference in values between tumor-bearing and tumor-resected nude mice is expressed as ** for \( P<0.01 \). ○, control nude mice; ▲, tumor-bearing nude mice; •, tumor-resected nude mice; \( \uparrow \), the day of tumor resection; †, death.

spleen index in LC-1-bearing nude mice. Plasma G-CSF in the control nude mice was undetectable (less than 0.01 ng/ml). In the LC-1-bearing nude mice with a tumor volume of 200 mm\(^3\), the level was markedly elevated, and was high in nude mice with tumors of various sizes. The WBC count was elevated in nude mice bearing tumors larger than 2000 mm\(^3\), and splenomegaly was initially observed at a tumor volume of 600 mm\(^3\). The spleen index was 2.9-fold greater than the control at maximum, and microscopically, many neutrophils and megakaryocytes were found in the spleen.

In the OCC-1-bearing nude mice, the WBC count, plasma G-CSF level and spleen index were determined at one point, two weeks after transplantation (data not shown). OCC-1-bearing nude mice
Tumor volume of 1900 ±400 mm³ were grouped as tumor-bearing and five other mice with the same tumor volume as tumor-resected. Fig. 4a shows the carcass body weight in the three groups, namely control, OCC-1-bearing and tumor-resected nude mice, according to the period after transplantation. In the control mice, the body weight was stable. All tumor-bearing mice died when the carcass body weight decreased to approximately 69% that of the control mice. In the tumor-resected group, the resections were carried out 11 days after transplantation, when the carcass body weight had decreased to 80% that of control nude mice and the tumor volume had reached approximately 1300 mm³. After tumor resection, the carcass body weight increased rapidly to the normal range. As shown in Table I, all LC-1-bearing mice died when the carcass body weight had decreased to 63% that of the controls. The tumors were resected 47 days after transplantation, when the body weight had decreased to 80% that of the control mice and the tumor volume was approximately 2000 mm³. After tumor resection, the carcass body weight increased rapidly to the normal range. As shown in Table I, the plasma calcium level was high in the tumor-bearing mice, but decreased and became normalized on the second day after tumor resection in the tumor-resected mice. The plasma PTHrP level in the tumor-resected mice was also decreased markedly on the second day after resection. Plasma G-CSF in the control mice was undetectable (less than 0.01 ng/ml), but the level in the tumor-bearing mice was high. In the tumor-resected group, G-CSF was undetectable on the second day after tumor resection (Table I), with normalization of the WBC count.

**Northern Blot Analysis**

Fig. 5 shows typical autoradiographs obtained by Northern blot analysis for mRNAs of PTHrP, G-CSF, M-CSF, IL-6 and LIF. Bands comparable to PTHrP mRNA with a molecular size of 1.7 and 2.3 kb, and G-CSF mRNA with a molecular size of 1.7 kb were detected in OCC-1 and LC-1 tumor tissues. IL-6 mRNA with a molecular size of 1.3 kb and LIF mRNA with a molecular size of 3.8 kb were detected in OCC-1 tumor tissue. A hybridizable band for M-CSF mRNA with a molecular size of 3.8 kb was detected in the OCC-1 tumor tissue. With the probe for β-actin mRNA, a 2.0 kb band was detected in each tumor sample. There were no detectable bands corresponding to the mRNAs of GM-CSF, IL-1α or IL-1β in the OCC-1 tumor tissue or mRNAs of M-CSF, GM-CSF, LIF or IL-6 in the LC-1 tumor tissue.

**Table I. Plasma Levels of Calcium, IAP, PTHrP and G-CSF in Tumor-bearing and Tumor-resected Nude Mice**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Tumor-bearing nude mice</th>
<th>Tumor-resected nude mice</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>2nd</td>
<td>4th or 5th</td>
</tr>
<tr>
<td>OCC-1</td>
<td>Calcium (mg/dl)</td>
<td>10.6±0.4</td>
<td>10.6±0.2</td>
</tr>
<tr>
<td></td>
<td>IAP (μg/ml)</td>
<td>190±16.0</td>
<td>200±65.0</td>
</tr>
<tr>
<td>LC-1</td>
<td>Calcium (mg/dl)</td>
<td>10.6±0.5</td>
<td>10.7±0.2</td>
</tr>
<tr>
<td></td>
<td>PTHrP (pmol/l)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G-CSF (ng/ml)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data represent means ±SD of plasma calcium and IAP levels. Plasma PTHrP and G-CSF levels were determined using pooled plasma in each group. 2nd, second day after tumor resection; 4th or 5th, fourth or fifth day after tumor resection; NT, not tested; IAP, immunosuppressive acidic protein; PTHrP, parathyroid hormone-related protein; G-CSF, granulocyte colony-stimulating factor.
Discussion

Two human squamous cell carcinoma cell lines, OCC-1 and LC-1, produced a triple paraneoplastic syndrome consisting of hypercalcemia, leukocytosis and cachexia. All three abnormalities disappeared rapidly after tumor resection, indicating their ectopic humoral nature. Yoneda et al. reported that nude mice bearing a human squamous cell carcinoma of the maxilla, MH-85, developed a triple paraneoplastic syndrome consisting of the same combination of hypercalcemia, leukocytosis and cachexia, and claimed that the symptoms were due to IL-6 produced by the MH-85 cells. However, in our model animals with a triple paraneoplastic syndrome, the symptoms were induced by concomitant production of multiple factors.

The hypercalcemia that developed in OCC-1- and LC-1-bearing nude mice could be explained by the production of PTHrP in the tumor cells. Northern blot analysis revealed that both cell lines expressed a large amount of PTHrP mRNA. Further studies on the plasma PTHrP level in tumor-bearing nude mice demonstrated that the elevation of the plasma PTHrP level was well correlated with that of the plasma calcium level, and that the level was as high as that in HHM patients. These findings indicated that PTHrP was the major factor responsible for hypercalcemia in these model animals.

Fig. 5. Expression of mRNAs for PTHrP, IL-6, LIF, G-CSF and β-actin in OCC-1 (Lane a) and LC-1 (Lane b) tumor tissues.

OCC-1- and LC-1-bearing nude mice showed marked leukocytosis. Autopsy studies of these animals revealed marked splenomegaly, and microscopic analysis revealed many neutrophils and megakaryocytes in the spleen. These findings indicated that both of the tumor cell lines can produce cytokines responsible for hematologic abnormalities. Northern blot studies demonstrated that OCC-1 and LC-1 tumor cells expressed G-CSF mRNA, and that the plasma G-CSF level was extremely high in the OCC-1- and LC-1-bearing mice. Therefore, G-CSF produced by the tumor cells may have been the major cause of leukocytosis in these animals.

Another remarkable feature common to these model animals was cachexia. We previously reported that nude mice bearing a melanoma cell line, SEKI, were good animal models for studies of cancer cachexia syndromes. The carcass body weight of SEKI-bearing nude mice had decreased to 74% that of control nude mice by day 25 after tumor transplantation. In the OCC-1 and LC-1-bearing nude mice, the carcass body weight decreased to a minimum of 69% and 63% that of the control mice, respectively, indicating that these model animals are useful for studies of cancer cachexia syndrome. It is unlikely that the severe hypercalcemia that developed in these animals was a cause of cachexia, since the body weight loss in tumor-bearing mice preceded the hypercalcemia. The disappearance of morbidity after tumor resection strongly suggests that these animal models can be placed in the category of ectopic humoral syndromes. In previous studies including ours, LIF and IL-6 were proposed to be candidate factors responsible for cancer cachexia in animal models. In the case of the OCC-1 cell line, Northern blot studies demonstrated that the tumor cells expressed mRNAs for LIF and IL-6, and sequential determination of the plasma LIF and IL-6 levels in tumor-bearing mice revealed that the levels increased gradually with tumor growth and the progression of body weight loss. Furthermore, the plasma IAP level was found to be elevated in OCC-1-bearing nude mice. Since it is now established that LIF and IL-6 act on liver cells, which in turn stimulate the production of acute-phase proteins such as IAP and C-reactive protein (CRP), these findings clearly indicated that both cytokines produced by cancer cells produce systemic changes in OCC-1-bearing nude mice in an endocrine fashion. However, in the LC-1-bearing nude mice, neither of these cytokines of IAP was elevated, suggesting the involvement of an unidentified factor with a mechanism of action independent of LIF and IL-6.

Animal models of triple paraneoplastic syndrome induced by multiple factors may be useful for studying the QOL of cancer patients. Bioactive sub-
stances produced concomitantly by cancer cells might induce multiple paraneoplastic syndromes. The present two animal models are examples of ectopic hormone and cytokine syndromes. Although multiple humoral syndromes are rarely detected in cancer patients, the present study suggests that tumor tissues have the ability to produce various factors responsible for multiple paraneoplastic syndromes. Future progress in modalities that may prevent the development of one paraneoplastic syndrome may make another paraneoplastic syndrome to become more prominent. Analysis of the sequence of events in paraneoplastic syndromes has revealed that the plasma levels of factors responsible for the syndromes begin to increase at an early stage in these animal models. These findings suggest that the development of such syndromes can be predicted by serial determination of the plasma factors responsible, which in turn should make it possible to improve the QOL of patients with advanced disease.

Acknowledgments

The authors thank Dr. Y. Ohnishi, Central Institute for Experimental Animals, for providing the OCC-1 and LC-1 tumor xenografts, and Ms. Y. Hasegawa and Ms. M. Ebinuma of our division for excellent technical assistance. This work was supported in part by a Research Grant from the Princess Takamatsu Cancer Research Fund, a Grant-in-Aid from the Ministry of Health and Welfare for the 2nd-term Comprehensive 10-Year Strategy for Cancer Control, Grants-in-Aid for Cancer Research (6-29) from the Ministry of Health and Welfare and by Special Coordination Funds from the Science and Technology Agency for Promotion of Science and Technology.

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