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Original Paper

Growth Stimulation of Non-small Cell Lung Cancer Xenografts by Granulocyte-macrophage Colony-stimulating Factor (GM-CSF)

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been suggested to be involved in the carcinogenesis of some types of tumours by autocrine or paracrine mechanisms. We examined GM-CSF/GM-CSF receptor (GM-CSFR) gene expression in 20 human non-small cell lung cancer (NSCLC) xenografts. The stimulatory effects of GM-CSF were examined using GM-CSF transgenic severe combined immunodeficient (SCID) mice (GM-Tg-SCID), which produce abundant human GM-CSF. A NSCLC xenograft (LC11-JCK), expressed GM-CSFR but not GM-CSF, and showed more rapid growth in GM-Tg-SCID than non-GM-CSF transgenic SCID mice (non-Tg-SCID). GM-CSF gene expression was detected in 48 of 90 (53%) primary NSCLC human specimens and GM-CSFR gene expression was detected in 42 specimens (47%). GM-CSF expression was detected in 13 of 30 squamous cell carcinoma specimens (43%) and GM-CSFR expression was detected in 10 specimens (33%). Patients with squamous cell carcinoma coexpressing GM-CSF and GM-CSFR showed significantly poorer prognosis than those expressing neither GM-CSF nor GM-CSFR ($P < 0.05$, Cox–Mantel test). These results suggest that GM-CSF can have a stimulatory effect on some NSCLC.

Key words: GM-CSF, non-small cell lung cancer, xenograft, transgenic mouse
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INTRODUCTION

GRANULOCYTE-MACROPHAGE colony-stimulating factor (GM-CSF) is a cytokine which stimulates the proliferation, differentiation, and function of myeloid progenitor cells. Several clinical trials of anticancer chemotherapy combined with recombinant human GM-CSF have been performed [1]. Whilst GM-CSF can lead to rapid neutrophil recovery, its use may promote tumour progression. Autocrine or paracrine mechanisms of action of GM-CSF have been reported in haematological and non-haematological malignancies [2–5]. However, the significance of autologous production of GM-CSF in clinical specimens is still unclear.

In this study, we examined growth stimulation by GM-CSF of human non-small cell lung cancer (NSCLC) xenografts using transplantation into GM-CSF transgenic severe combined immunodeficient (SCID) mice. We also examined GM-CSF and GM-CSF receptor (GM-CSFR) gene expression in 90 primary NSCLC and discuss their clinicopathological significance.

MATERIALS AND METHODS

Tumour specimens

Twenty human NSCLC xenografts transplanted in SCID mice, passages 10–20, were supplied by the Central Institute for Experimental Animals (Kawasaki, Kanagawa, Japan). NSCLC specimens were obtained from 90 primary lesions resected surgically from patients who gave their informed consent. Viable cancers were used, avoiding necrotic or

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degenerative regions. Tissues were rapidly frozen and stored at −80°C until analysis. Total cellular RNA was prepared from the frozen specimens by standard procedures. Surgical specimens were also processed for routine histopathological analysis. Histopathologically, the specimens were classified as adenocarcinoma, 54; squamous cell carcinoma, 30; large cell carcinoma, six; stage I, 49; stage II, nine; stage IIIa, 28; stage IIIb, four. This experiment was approved by the medical ethical committee of Tokai University School of Medicine.

GM-CSF and GM-CSFR gene expression

GM-CSF and GM-CSFR gene expression were examined by reverse transcription–polymerase chain reaction (RT–PCR, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min). The following primers were used: TCTCCTGAACCTGAGTAGAG (GM-S, 131–150) and GGATGACAAGCAGAAAGTCC (GM-A, 392–411) for GM-CSF; CCTTCTGCTCTGTGAGTTACC (GMR-S, 183–203) and GGGTATTCTTTCTGTGGACG (GMR-A, 930–949) for GM-CSFR. Primers GMR-S and GMR-A can amplify cDNA fragments specific for the α chain of GM-CSFR. Blots of PCR fragments (Zeta-Probe, Biorad, California, U.S.A.) were detected by hybridisation with a photochemically labelled cDNA probe (enhanced chemiluminescence (ECL) Amersham, Buckinghamshire, U.K.).

GM-CSF stimulation of NSCLC xenograft in vivo

GM-CSF transgenic SCID mice (GM-Tg-SCID) established and maintained at the Central Institute for Experimental Animals [6] were used for the investigation of the influence of GM-CSF stimulation on the growth of NSCLC xenografts. Serum levels of GM-CSF were 2–10 ng/ml in the GM-Tg-SCID and not detectable in non-Tg-SCID mice. Human lung cancer xenografts LC11-JCK (GM-CSF−/GM-CSFR+) and LC20-JCK (GM-CSF+/GM-CSFR+) were used. The xenografts were subcutaneously transplanted into seven GM-Tg-SCID mice (10–12 weeks old) and seven non-transgenic littermates (non-Tg-SCID). Tumour size was measured sequentially and mice were sacrificed 4 weeks after transplantation. The tumour volume (V) was calculated by the equation, $V = 1/2 \times A \times B^2$ in which A and B are the experimental measurements in mm of the length and width, respectively. Growth of the tumour xenografts was measured by the relative tumour volume (RV), which was expressed as $RV = V_x/V_0$ where V_x is the tumour volume at day x and V_0 is the tumour volume at day 7. When the animals showed severe wasting and were apparently moribund due to tumour growth, they were killed according to UKCCCR guidelines [7]. Before sacrifice, 5-bromo-2′-deoxyuridine (BrdU, 0.5 mg/10 g) was administered intraperitoneally. Excised tumour specimens were fixed in 10% formaldehyde and embedded in paraffin, and sections were immunostained with anti-BrdU monoclonal antibody (MAS 250b) (Sera-Lab Ltd, Crawley Down, Sussex, U.K.). BrdU-positive cancer cells were counted at ×400 magnification, and the BrdU labelling index (LI) was calculated as a percentage in 1,000 cancer cells [8].

Statistical analysis

Survival rate of the patients with lung cancer was estimated using Kaplan–Meier life tables, and the curves were analysed for significance by the Cox–Mantel test with $P < 0.05$ taken to indicate significance. The Mann–Whitney U test was used to estimate the differences between two groups.

RESULTS

GM-CSF and GM-CSFR gene expression in the NSCLC xenografts

Twelve of 20 (60%) NSCLC xenografts were positive for GM-CSF gene expression, and eight (40%) expressed GM-CSFR (Figure 1a, b). Six xenografts (LC20-JCK, LC52-JCK, LC53-JCK, LC54-JCK, LC55-JCK and LC58-JCK) showed coexpression of GM-CSF and GM-CSFR. LC11-JCK and LC29-JCK xenografts showed GM-CSFR expression, but did not show GM-CSF expression.

Growth stimulation of the NSCLC xenograft by GM-CSF in vivo

To clarify the influence of GM-CSF stimulation on the growth of NSCLC, we studied the growth of NSCLC xenografts positive for GM-CSFR expression in GM-Tg-SCID. The xenograft LC11-JCK, expressing GM-CSFR but not GM-CSF, showed a significantly increased LI value ($n = 7$, LI: 29.8 ± 3.84) compared with xenografts in non-Tg-SCID mice ($n = 7$, LI: 22.6 ± 1.20, $P = 0.006$, Mann–Whitney U test, Figure 2a, b). The xenograft LC11-JCK also showed significantly more rapid growth in GM-Tg-SCID than non-Tg-SCID mice (on day 28, $P = 0.021$, $n = 7$ Mann–Whitney U test, Figure 3). The xenograft LC20-JCK, coexpressing GM-CSF and GM-CSFR, showed no significant difference in growth between GM-Tg-SCID and non-Tg littermates (data not shown). The xenografts (GM-CSF+/GM-CSFR−, GM-CSF−/GM-CSFR−) showed no significant difference in growth in GM-Tg-SCID (data not shown).

GM-CSF and GM-CSFR gene expression in primary NSCLC

Forty-eight of 90 NSCLC (53%) expressed GM-CSF, and 42 specimens (47%) expressed GM-CSFR (Figure 4a, b). GM-CSF gene expression was detected in 31 of 54 adenocarcinomas (57%), 13 of 30 squamous cell carcinomas (43%) and four of six large cell carcinomas (67%). GM-CSFR gene expression was detected in 29 of 54 adenocarcinomas (54%), 10 of 30 squamous cell carcinomas (33%) and three of six large cell carcinomas (50%). Twenty-six (20 adenocarcinomas, five squamous cell carcinomas and one large cell carcinoma)

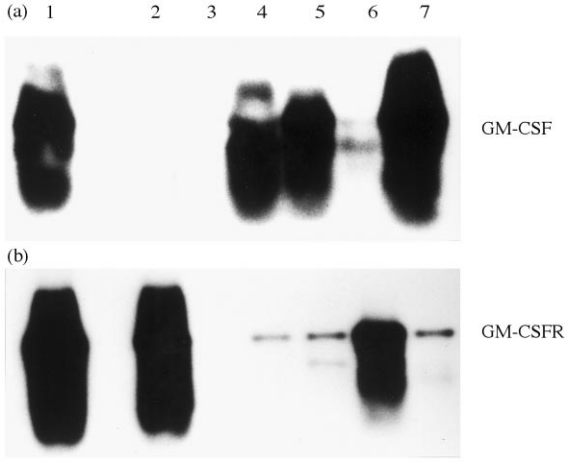

Figure 1. (a) Granulocyte-macrophage colony stimulating factor (GM-CSF) and (b) GM-CSF receptor (GM-CSFR) gene expression in human lung cancer xenografts: 1, CHU-2 for GM-CSF control and human placental RNA for GM-CSFR control; 2, LC11-JCK; 3, LC17-JCK; 4, LC20-JCK; 5, LC53-JCK; 6, LC54-JCK; 7, LC55-JCK.

of the 90 (29%) NSCLC coexpressed both GM-CSF and GM-CSFR genes.

GM-CSF gene expression was detected in 26 stage I (53%), seven stage II (78%), 12 stage IIIa (43%) and three stage IIIb (75%) tumours, and GM-CSFR gene expression was detected in 26 (53%), four (44%), 10 (36%) and two (50%), respectively.

Prognosis of NSCLC coexpressing GM-CSF and GM-CSFR genes

Follow-up of patients ranged from 1 to 69 months (mean 31.2 months) in the 90 NSCLC patients. The 5-year survival rate was 60.8% for patients with NSCLC expressing GM-CSF ($n = 48$) and 66.7% for those with GM-CSF-negative tumours ($n = 42$). The NSCLCs coexpressing GM-CSF and GM-CSFR (26/90) did not show significantly poorer prognosis than those without coexpression of these genes (5-year survival rate was 64.3%).

The 13 squamous cell carcinomas expressing GM-CSF showed a poorer prognosis than the 17 squamous cell carcinomas without GM-CSF gene expression, and the prognosis was significantly worse in squamous cell carcinomas coexpressing GM-CSF and GM-CSFR ($n = 5$) than in those not expressing these genes ($n = 12$) ($P < 0.05$, Cox–Mantel test, Figure 5).

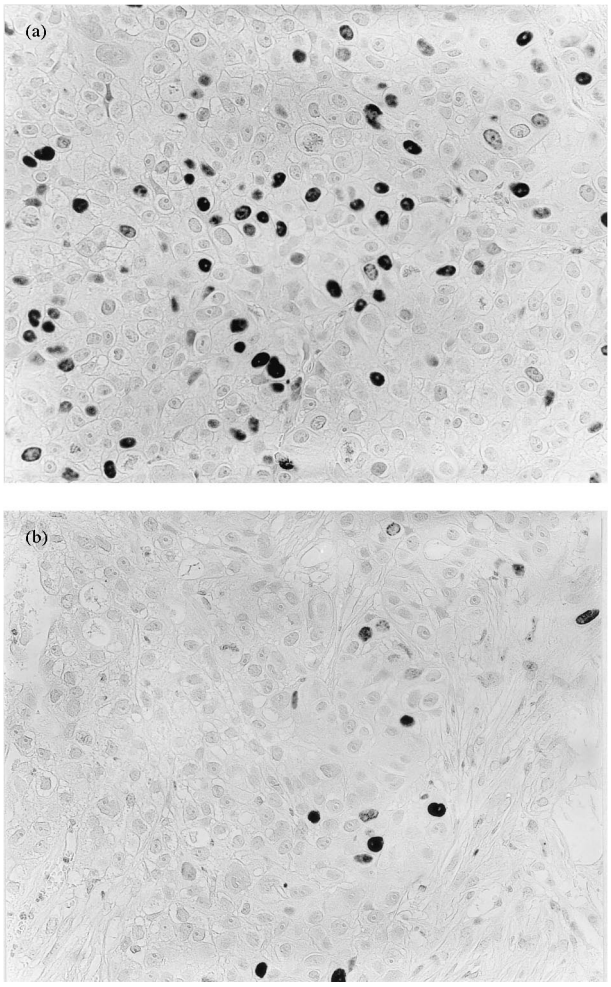

Figure 2. 5-bromo-2′-deoxyuridine (BrdU) staining of non-small cell lung cancer (NSCLC) xenografts LC11-JCK in (a) GM-Tg-SCID and (b) Non-Tg-SCID (×300). The labelling index was calculated as a percentage of BrdU-positive cells in 1,000 cancer cells.

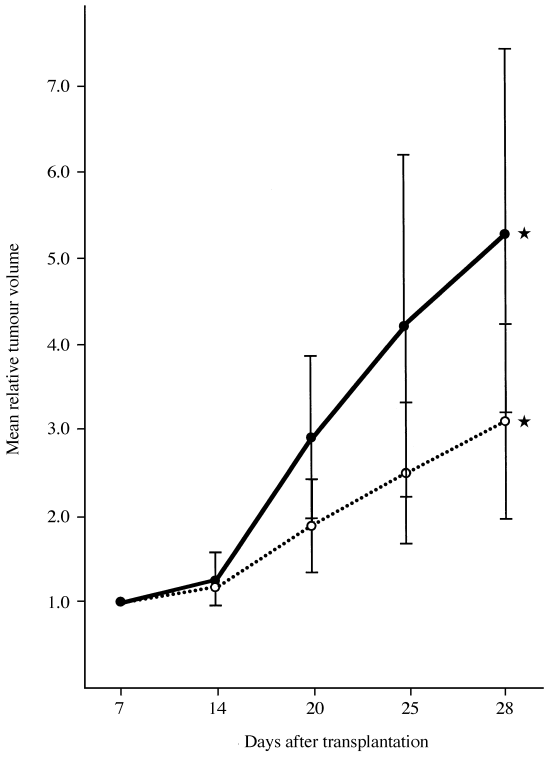

Figure 3. The xenograft LC11-JCK, expressing granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) but not GM-CSF, showed more rapid growth in GM-Tg-SCID (solid line) than non-Tg-SCID (broken line). *P=0.021, Mann–Whitney U test.

Figure 4. (a) Granulocyte-macrophage colony stimulating factor (GM-CSF) and (b) GM-CSF receptor (GM-CSFR) gene expression in human non-small cell lung cancer (NSCLC) specimens: 1, CHU-2 for GM-CSF control and human placental RNA for GM-CSFR control; 2–6, NSCLC specimens.

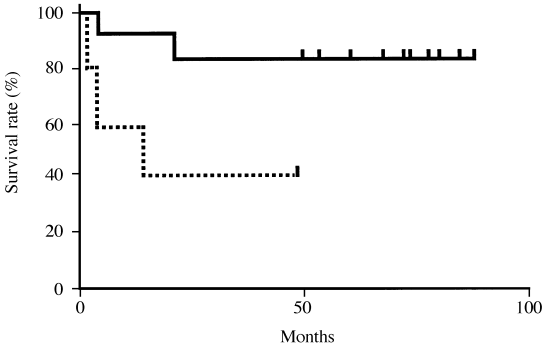

Figure 5. Survival curve of squamous cell carcinomas. Patients with tumours coexpressing granulocyte-macrophage colony stimulating factor (GM-CSF) and GM-CSF receptor (GM-CSFR) (broken line) showed poorer prognosis than those not expressing these genes (solid line, P<0.05, Cox–Mantel test).

No differences were seen for adenocarcinomas, and the limited number of large cell carcinomas precluded statistical comparisons.

DISCUSSION

Autonomous production of GM-CSF has been reported in some non-haematological neoplasms [9, 10] and certain cancer cells respond to exogenous administration of GM-CSF *in vitro* [11]. However, the role of GM-CSF in tumour progression is still unclear. When the human lung cancer xenograft LC11-JCK (GM-CSF – /GM-CSFR +) was transplanted into GM-Tg-SCID, growth was augmented as compared with that in non-Tg-SCID littermates. BrdU incorporation analysis confirmed the augmentation of LC11-JCK growth at the level of the cell cycle. However, when LC20-JCK (GM-CSF + /GM-CSFR +) was transplanted into GM-Tg-SCID, the tumour did not show any significant augmentation of growth. These results suggest that GM-CSFR expressed on NSCLC is functional only under certain conditions. Further studies using anti-GM-CSF or anti-GM-CSFR antibodies are required to clarify the possibility of the existence of an autocrine loop of GM-CSF in NSCLC xenografts.

We demonstrated GM-CSF gene expression in 53% of NSCLCs and GM-CSFR gene expression in 47%. In squamous cell carcinoma (30 cases), the patients with GM-CSF and GM-CSFR coexpression (5 cases) showed poorer prognosis than those without expression of these genes (12 cases), whilst GM-CSF alone was not a significant prognostic factor. It appears that the putative autocrine loop of GM-CSF is significant in the progression of only some types of pulmonary squamous cell carcinoma.

GM-CSF is considered to be a useful support cytokine for high-dose anticancer chemotherapy, and several clinical trials are currently underway [12–14]. GM-CSF has the potential to be an antineoplastic cytokine by induction of differentiation of macrophages and monocytes, enhancement of their natural cytotoxicity, and augmentation of antibody-dependent cellular cytotoxicity [15]. However, GM-CSF may have potential risks for cancer patients, especially in patients with tumours expressing GM-CSFR, since our results suggest that GM-CSF stimulation may promote tumour progression of NSCLC.

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