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Novel models of cancer-related anemia in mice inoculated with IL-6-producing tumor cells

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ABSTRACT

We established models of cancer-related anemia in mice from subcutaneous inoculation of two IL-6-producing cancer cell lines, human lung cancer cell line LC-06-JCK and murine colon26 clone 5 colon cancer cells. In both models, elevated levels of IL-6 were detected in sera and hemoglobin levels significantly decreased compared with non-tumor-bearing mice. In the LC-06-JCK model, serum albumin levels also decreased with elevated levels of human IL-6 in sera. On the other hand, serum levels of EPO increased, although anemia developed and did not improve. The development of cancer-related anemia was prevented by the administration of a rat anti-mouse IL-6 receptor antibody, MR16-1, in the LC-06-JCK model. It is therefore suggested that IL-6 causes anemia independent of a reduction in EPO levels. Our preclinical models should be useful for exploring new modalities for the treatment of cancer-related anemia.

It has been reported that the incidence of cancer-associated anemia (Hb < 12.0 g/dL) was 30.7% in newly-diagnosed, previously untreated cancer patients, and that low hemoglobin levels correlate with poor performance scores in cancer patients (5). Anemia in cancer patients cannot be simply accounted for by conventional explanations such as blood loss, hemolysis, or nutritional deficiencies. Cancer-related anemia may be described as a cytokine-mediated disorder of erythropoiesis. Over-expression of certain inflammatory cytokines can suppress the erythroid progenitors, impair iron utilization, and reduce erythropoietin (EPO) production.

Serum levels of the inflammatory cytokine IL-6 have been reported to be elevated in patients with breast, colorectal, and lung cancers (3, 7, 14), and to negatively correlate with hemoglobin levels in cancer patients (2, 6). Furthermore, Nieken *et al.* reported that administration of recombinant human IL-6 as immunotherapy caused anemia in cancer patients (10).

In the present study, we demonstrated that hemoglobin levels decreased in animal models bearing IL-6-producing tumor tissues such as LC-06-JCK human lung (12) and colon26 clone 5 murine colon cancers (4) and, furthermore, that a rat anti-IL-6 receptor antibody prevented the induction of cancer-related anemia in the LC-06-JCK model. These results suggest that IL-6 could be a mediator of cancer-related anemia.

MATERIALS AND METHODS

Cancer cells. Human cancer cell line LC-06-JCK (lung clear cell carcinoma) and PAN-07-JCK (pancreatic adenocarcinoma) were obtained from the Central Laboratory Animal Research Center (Kawasaki, Japan) and maintained *in vivo* in nude mice (BALB/c nu/nu). Colon26 clone 5 was established from murine colon26 carcinoma, as previously reported (4).

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Animal models. Five-week-old male nude rats (F344/N Jcl-nu) were purchased from Clea (Kawasaki, Japan) and seven-week-old male nude mice (BALB/c nu/nu) and male CDF1 mice were purchased from Charles River Japan (Yokohama, Japan). Tumors of LC-06-JCK and PAN-07-JCK grown in nude mice were resected, and small pieces were subcutaneously inoculated into nude rats or nude mice, as previously described (12). Colon26 clone 5 cells were maintained *in vitro* with RPMI 1640 containing 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. The cells were treated with 0.05% trypsin and 0.02% EDTA to obtain a single-cell suspension; 10^6 cells were subcutaneously inoculated into CDF1 mice. Rats and mice were fed rodent chow and water *ad libitum*. All studies were conducted according to the guidelines for the care and use of laboratory animals set in Chugai Pharmaceutical Co., Ltd.

Measurement of serum cytokines, albumin and hemoglobin. Serum levels of cytokines and albumin were determined using commercially available ELISA kits for human IL-6 (R&D Systems, Minneapolis, MN, USA), murine IL-6 (Pierce, Rockford, IL, USA), murine EPO (R&D), and murine albumin (Shibayagi, Gunma, Japan). Hemoglobin levels were determined by an automated hematology analyzer (KX-21NV; Sysmex, Kobe, Japan).

Administration of rat anti-murine IL-6 receptor monoclonal antibody. To examine the role of IL-6 in the induction of anemia in mice bearing IL-6-producing tumors, we used a rat anti-murine IL-6 receptor monoclonal antibody, MR16-1 (11). Although MR16-1 is specific for the murine IL-6 receptor, it can block the biological effects of IL-6 derived not only from mice also from humans in murine models because human IL-6 can induce the proliferation of murine cells (13). MR16-1 was intraperitoneally administrated at a dose of 20 mg/kg once a week in the LC-06-JCK model, starting 3 weeks after inoculation of the cancer cells.

Administration of humanized anti-PTHrP monoclonal antibody. The humanized anti-PHTrP monoclonal antibody raised against the N-terminal 34 amino acids of human PTHrP was intravenously administrated at 3 mg/kg in the LC-06-JCK and PAN-07-JCK models, as previously reported (12).

RESULTS

We measured the hemoglobin levels in nude rats bearing human lung cancer LC-06-JCK and human pancreatic cancer PAN-07-JCK, both of which have been previously reported to produce PTHrP and to cause cachexia with wasting in nude rats (12). Hemoglobin levels and red blood cell count were reduced in nude rats bearing LC-06-JCK but not in nude rats bearing PAN-07-JCK (Table 1). Anemia in the LC-06-JCK model was not improved by administration of the humanized anti-human PTHrP monoclonal antibody (Table 1) that has been reported to prevent cachexia in both models (12).

We further investigated cancer-related anemia in murine models because rat anti-IL-6 receptor monoclonal antibody MR16-1 is specific to mice, not to rats. Hemoglobin levels decreased and human IL-6 levels were elevated in nude mice bearing LC-06-JCK on days 51, 65 and 72 after tumor inoculation (Fig. 1) but not in mice bearing PAN-07-JCK (data not shown). Furthermore, in nude mice bearing LC-06-JCK, serum albumin levels significantly decreased, whereas murine EPO levels were significantly elevated during the period of anemia (Fig. 1). Serum levels of murine IL-6 were below the detection limit during the testing period in the LC-06-JCK model (data not shown).

We also measured hemoglobin levels in mice bearing colon26 clone 5, which has been reported to be a non-cachectic subclone of the murine colon cancer cell line, colon26, and to produce murine

Table 1 *Anemia and cachexia models in nude rats bearing human cancer cells*

	LC-06-JCK		Normal	PAN-7-JCK		Normal
Parameters	Vehicle	PTHrP Ab	rats	Vehicle	PTHrP Ab	rats
Body weight (g)	185	274	294	222	267	286
WBC ($\times 10^2/\mu L$)	406	247	172	204	143	94
RBC ($\times 10^4/\mu L$)	616	604	912	981	880	921
Hb (g/dL)	10.8	10.2	15.4	16.8	15.1	15.9
PLT ($\times 10^4/\mu L$)	69.9	69.0	58.4	51.8	48.7	50.4

Results indicated are mean values of parameters in 8 rats/group. WBC, white blood cells; RBC, red blood cells, Hb: hemoglobin, PLT: platelets. Nude rats bearing tumors received 3 mg/kg of anti-PTHrP antibody or saline (vehicle).

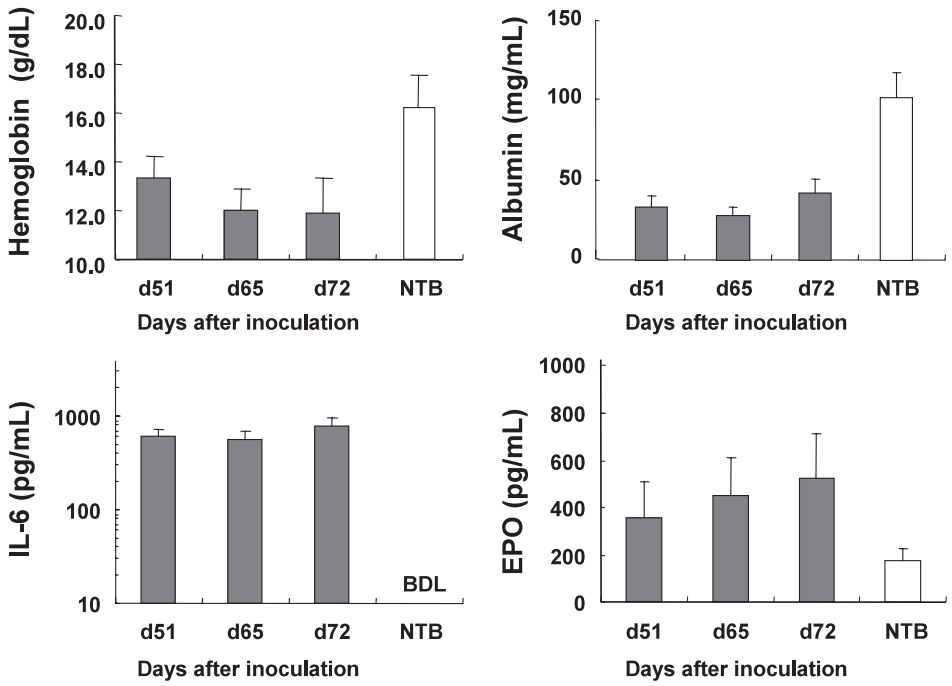

Fig. 1 Time courses of hemoglobin levels and serum levels of human IL-6, murine albumin and murine EPO in nude mice bearing human lung cancer LC-06-JCK. Results are the mean ± SD of 5 mice. BDL: below the detection limit (15 pg/mL). NTB: non-tumor-bearing mice.

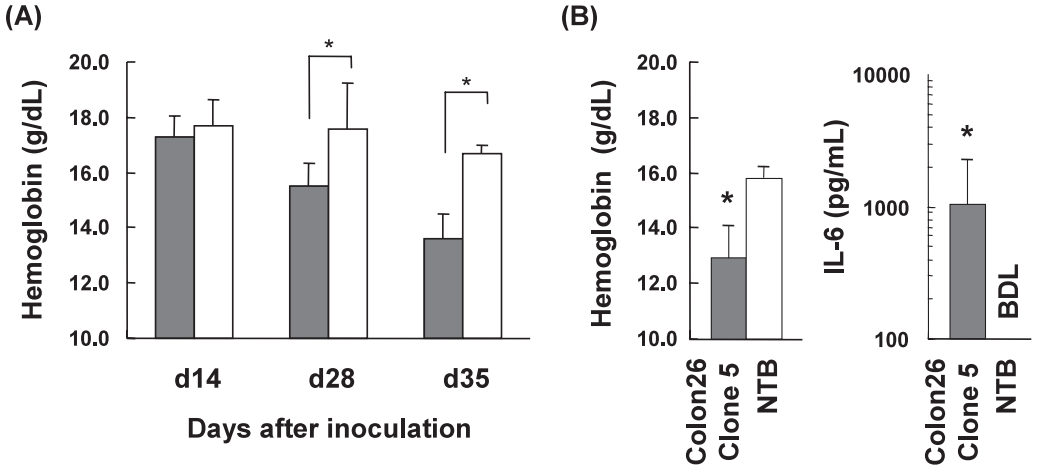

Fig. 2 Hemoglobin levels and serum levels of murine IL-6 in mice bearing colon26 clone 5. Parameters were measured 7 weeks after inoculation with clon26 clone 5 cells. (A) Results are the mean ± SD of 5 or 8 mice. Closed bars, mice bearing colon26 clone 5 tumors (n = 8/group); open bars, non-tumor-bearing mice (n = 5/group). *$P < 0.05$ (Wilcoxon test). (B) Results are the mean ± SD of 5 mice. BLD, below the detection limit (50 pg/mL); NTB, non-tumor-bearing mice. *: $P < 0.05$ (Wilcoxon test).

IL-6 (4). In mice bearing colon26 clone 5, hemoglobin levels significantly decreased on day 28 and day 35 after inoculation with cancer cells (Fig. 2A). On day 35, hemoglobin levels remained low and serum levels of murine IL-6 were significantly elevated compared with non-tumor-bearing mice (Fig. 2B).

Rat anti-murine IL-6 receptor antibody MR16-1 was intraperitoneally administered at a dose of 20 mg/kg once a week in the LC-06-JCK model, starting 3 weeks after inoculation of the cancer cells. MR16-1 significantly inhibited the decrease in hemoglobin levels (Fig. 3). In the LC-06-JCK

model, decreases in serum albumin were also prevented by administration of MR16-1 (Fig. 4). Elevated serum levels of human IL-6 and murine EPO were not significantly affected by treatment with MR16-1. Murine IL-6 was not detected in non-tumor-bearing mice or in tumor-bearing mice regardless of MR16-1 treatment. A lack of detection of murine IL-6 by ELISA is not due to the presence of MR16-1 in serum because MR16-1 binds to murine IL-6 receptors, not murine IL-6.

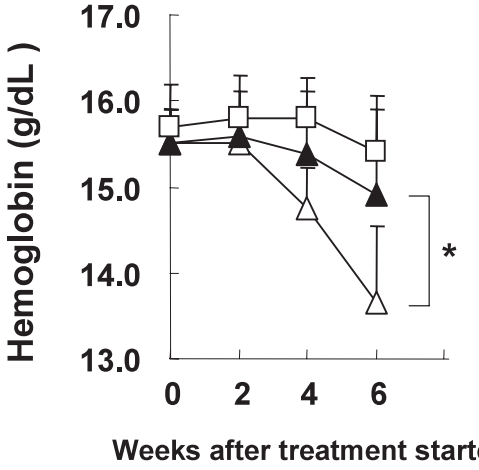

Fig. 3 Preventive effect of a rat anti-murine IL-6 receptor antibody, MR16-1, against cancer-related anemia in the LC-06-JCK model. Results are the mean ± SD for 8 mice. Open squares, non-tumor-bearing mice; open triangles, tumor-bearing mice treated with normal rat IgG; closed triangles, tumor-bearing mice treated with MR16-1. Rat IgG or MR16-1 was intraperitoneally administered at a dose of 20 mg/kg once a week for 6 weeks. Treatment was initiated 3 weeks after inoculation with LC-06-JCK. *: $P < 0.05$ (Wilcoxon test).

DISCUSSION

In the present study, we established murine models of cancer-related anemia in which serum hemoglobin levels decreased after inoculation with IL-6-producing cancer cells. It has been reported that administration of recombinant human IL-6 caused anemia in cancer patients (10). Furthermore, it has been reported that serum levels of IL-6 negatively correlated with hemoglobin levels in cancer patients (2, 6). In our cancer-related anemia models, serum levels of IL-6 increased and hemoglobin levels decreased. Serum albumin also decreased in the LC-06-JCK model. IL-6 is a member of the family of inflammatory cytokines and induces acute phase reactions such as reduction of serum albumin. Clinically, low levels of serum albumin predict poor prognosis for cancer patients. The reduction of serum albumin does not merely imply malnutrition in cancer patients, and IL-6 would have an adverse impact on prognosis in such patients. IL-6 has been reported to be a cachectic factor in murine models, where serum levels of IL-6 are elevated and administration of anti-IL-6 antibody prevents the development of cancer cachexia (4). In the present study, anemia was observed in mice bearing a cachectic human lung cancer, LC-06-JCK, as well as in mice bearing the non-cachectic colon26 clone 5. Therefore, it is suggested that IL-6 causes cachexia and anemia through independent mechanisms.

Recently, it has been reported that IL-6 mediates hypoferremia by inducing the iron regulatory hormone hepcidin (8), which has been proposed to be an important factor in the pathogenesis of the anemia of chronic disease including cancers (1). Hepcidin production has been reported to be induced by IL-6 in human hepatocyte cultures, indicating that

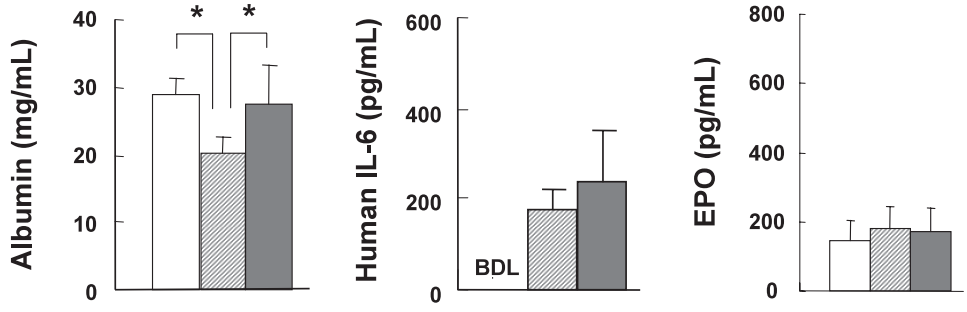

Fig. 4 Effects of MR16-1 on serum levels of albumin, human IL-6 and murine EPO in the LC-06-JCK model. Results are the mean ± SD for 8 mice. BDL, below the detection limit (15 pg/mL). Open bars, non-tumor-bearing mice; hatched bars, tumor-bearing mice treated with normal rat IgG; closed bars, tumor-bearing mice treated with MR16-1. Rat IgG or MR16-1 was intraperitoneally administered at a dose of 20 mg/kg once a week. The treatment was initiated 3 weeks after inoculation with LC-06-JCK. All parameters were measured in mice after being treated for 4 weeks. *: $P < 0.05$ (Wilcoxon test).

hepcidin induction from inflammation is a type II acute-phase response, whereas ferritin production is induced by IL-1 and TNF-α and so is classified as a type I acute-phase response (9). In our cancer anemia models, serum levels of EPO were elevated, even though anemia was sustained. We speculate that hepcidin would disturb the erythropoiesis and decrease hemoglobin levels, which would induce EPO production in the anemia models with elevated levels of tumor tissue-derived IL-6; however, in the present study, we have not demonstrated the role of hepcidin in cancer-related anemia.

MR16-1 is a rat monoclonal antibody specific to the murine IL-6 receptor and does not bind to the human IL-6 receptor. Therefore, MR16-1 cannot block human IL-6 receptors on human cells. Indeed, tumor growth of LC-06-JCK in mice was not significantly affected by MR16-1 treatment in the present study (data not shown). On the other hand, administration of MR16-1 prevented the development of cancer-related anemia in nude mice bearing human cancer LC-06-JCK. MR16-1 also prevented a decrease in serum albumin. Therefore, it can be inferred that MR16-1 would block murine IL-6 receptors on murine host cells from binding to the human IL-6 produced by LC-06-JCK tumors and thus prevent the development of anemia. However, in the colon26 clone 5 model, MR16-1 at a dose of 20 mg/kg once a week showed no significant prevention of the development of anemia from administration of MR16-1 starting 2 weeks after inoculation of colon26 clone 5 (data not shown). Administration of MR16-1 might need to be started earlier or at higher doses to prevent anemia from developing in this model.

In the present study, we demonstrated that IL-6 caused cancer-related anemia in two mouse models, LC-06-JCK and colon26 clone 5. Furthermore, we have shown that the development of cancer-related anemia was prevented by blocking the IL-6 receptors on murine host cells with a rat anti-mouse IL-6 receptor antibody. Although the mechanisms by which IL-6 causes cancer-related anemia are not yet clear, we are investigating the involvement of hepcidin. Our preclinical models should be useful for investigating the roles of IL-6 in cancer-related anemia and for exploring new modalities for the treatment of cancer-related anemia.

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