Use of recombinant human soluble TNF receptor in anorectic tumor-bearing rats

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1Surgical Metabolism and Nutrition Laboratory, Department of Surgery, University Hospital, State University of New York Health Science Center, 13210; 2Department of Mathematics, Syracuse University, Syracuse, New York 13244; 3Department of Surgery, College of Medicine, University of Florida, Gainesville, Florida 32610; 4Amgen, Incorporated, Thousand Oaks, California 91320; and 5Department of Clinical Medicine, University of Rome “La Sapienza,” Rome, Italy 00185

Torelli, Giovanni F., Michael M. Meguid, Lyle L. Moldawer, Carl K. Edwards III, Hyune-Ju Kim, Janna L. Carter, Alessandro Laviano, and Filippo Rossi Fanelli. Use of recombinant human soluble TNF receptor in anorectic tumor-bearing rats. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R850–R855, 1999.—With progression of tumor growth, rats demonstrate anorexia and reduced food intake, a function of meal number and meal size. Tumor necrosis factor-α (TNF-α), a recognized anorectic agent, reacts with two different receptors (type I: 55 kDa; type II: 75 kDa). We used a dimeric, pegylated 55-kDa TNF receptor construct to test its effects on food intake, meal number, and meal size, which were continuously measured with a rat eater meter in 16 Fischer 344 male rats injected with 10⁶ viable methylicholanthrene cells. When anorexia developed, rats received a subcutaneous injection of either 0.25 mg/kg body wt of soluble TNF receptor construct (study) or vehicle (tumor-bearing control). Before TNF inhibitor injection, no differences were observed in food intake, meal number, or meal size between the two groups. After the TNF inhibitor injection, study vs. control rats significantly improved food intake as a result of an increase in meal number and meal size. Rats also showed a significant improvement in body weight. These data suggest that TNF-α, in addition to other cytokines, contributes to the anorexia of tumor growth, probably mediated via the hypothalamus.

cancer anorexia; food intake regulation; feeding behavior; tumor necrosis factor-α

IN TUMOR-BEARING RATS, anorexia and reduced food intake are frequently observed. When translated to the human situation, this scenario contributes to the development of malnutrition and to a worsening of the overall chances of survival (25).

In the pathogenesis of cancer anorexia, both central and peripheral factors participate in determining the reduction of food intake during tumor growth (23). Cytokines play a major role by modulating hypothalamic feeding sites (36), modifying neurotransmitter concentrations (27), and influencing brain catecholaminergic and serotonergic systems. Among them, tumor necrosis factor-α (TNF-α) is a well-recognized anorectic agent (22, 36).

TNF-α, produced by blood monocytes and tissue macrophages in response to tumors (17), as well as by the tumor itself (13), can interact with two distinct surface receptors with molecular weights of 55 and 75 kDa (32). The intracellular domains of the p55 (type I) and p75 (type II) TNF receptors differ, suggesting distinct signal transduction pathways. In vivo studies showed improvement in the inflammatory symptoms of rheumatoid arthritis during a 3-mo trial using the soluble TNF receptor p75 linked to the Fc portion of human IgG (18), whereas stimulation of the p55 TNF receptor induced activation of coagulation and fibrinolysis in baboons (34).

TNF-α acts directly on the central nervous system to produce its anorectic effect (5, 23, 36) by crossing the blood-brain-barrier (7). Whether administered centrally or peripherally, TNF-α suppresses food intake in a dose-dependent manner (2). The central mechanisms for the action of TNF-α appear to be related to its modulatory effects on neural activity of glucose-sensitive neurons within the ventromedial nucleus of the hypothalamus (VMN) (8) and the lateral hypothalamic area (LHA) (24), and to the stimulation of hypothalamic PGE₂ synthesis (4), which in turn stimulates the release of corticotropin-releasing factor associated with an anorectic effect (33).

The mode of anorexia occurrence in tumor-bearing rats may also further strengthen the link between TNF-α and cancer-associated anorexia. Daily food intake (FI) is a function of the number of meals per day (MN) and the size of each meal (MZ) (i.e., FI = MN × MZ). Thus the reduction of FI during anorexia can be accomplished via a reduction of MN and/or MZ, simultaneously or via different temporal occurrences. The aims of this study were 1) to measure FI and feeding indexes of MN and MZ at the onset of cancer anorexia and 2) to test the effect of a TNF inhibitor, a soluble dimeric, pegylated 55-kDa TNF receptor construct, to ascertain whether its subcutaneous administration would modulate feeding pattern and/or reduce anorexia by increasing FI, thereby attenuating body weight loss.

MATERIALS AND METHODS

Animals

The experiments were approved by the Committee for the Humane Use of Animals at the State University of New York Health Science Center at Syracuse and were in accordance

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Male Fischer 344 rats (n = 16; Taconic, Georgetown, NY), with an initial weight of 240–260 g were housed in rat colony cages for 10 days to acclimate them to the constant study surroundings: 12:12-h light-dark cycle (lights off 1700–0500), 25 ± 1°C room temperature, and 45% relative humidity. Rats had free access to fresh, coarsely ground chow (Diet #5008; Ralston Purina, St. Louis, MO) and tap water.

General Procedures, Definition of Anorexia, and Study Design

After the acclimatization period, rats were placed in individual cages equipped with the automated computerized rat eater meter (ACREM; Fig. 1). Purina rat chow (Diet #5008; Ralston Purina) and tap water were available ad libitum. The ACREM consists of commercially available metabolic cages, in which the supplied feeding cup at the end of the feeding tunnel is replaced by an electronic scale balance and two photoelectric cells centered above the food dish. A real-time remote computerized data collection device integrates feeding activity as measured by the electronic scale and the photo-cells. The ACREM characterizes feeding activity of the rat by monitoring access to the food cup. A meal is defined as a bite consumed per 24 h; FI (g/day) = amount of food consumed per 24 h; MN (meals/day) = total number of meals per 24 h; and MZ (g/meal) = total amount of food consumed per each meal during 24 h. The ACREM also permits the discrimination between light- and dark-phase eating activity; however, for reasons of simplicity, we elected to show only the 24-h data. Ten days after being placed in the ACREM cages, rats received a subcutaneous injection of 0.5 ml of 406 trypan blue viable methylcholanthrene sarcoma cells into the right flank. The tumor cell suspension was prepared according to Madden and Burk (14). The given dose produces a tumor which becomes palpable ~9 days after inoculation and induces anorexia at a mean of 18 days after inoculation (1, 8, 11).

The rats’ body weights were measured daily. Tumor size was also measured daily, and tumor volume and weight were calculated with the formula for a prolate spheroid (V = 1/2ab², where a is the longer and b the shorter dimension).

Tumor-bearing rats were defined as being anorectic after three consecutive days in which each rat’s food intake was reduced by at least 1 g/100 g body wt compared with the mean daily food intake of the pretumor inoculation period, according to a modification of the definition of Chance et al. (3).

On the day the rats were diagnosed to be anorectic (day 0), they were randomly assigned to receive either the soluble pegylated 55-kDa TNF receptor construct or normal saline, the vehicle. Study rats received a subcutaneous injection of 0.25 mg/kg body wt of TNF receptor dissolved in 500 µl of saline into the left flank, whereas control rats received 500 µl of saline. The dimeric, pegylated soluble TNF receptor (p55) construct was obtained from Amgen (Thousand Oaks, CA). Comprised of two extracellular domains of the human p55 TNF receptor covalently linked with polyethylene glycol, this compound has extended biological half-life and neutralizes human, baboon, mouse, and rat TNF-α (29, 26). The dose employed was based on earlier studies with the same construct in rodent models of endotoxin shock and experimentally induced arthritis (26, 9).

Statistical Analysis and Data Handling

Data were analyzed with t-tests, general linear models, and mixed-effect models for FI, MN, MZ, and body weight. SAS, statistical software from the SAS Institute, was used for all analysis. The t-tests were used to test for daily mean differences for each of the feeding indexes. In examining the group, the day, their interaction, and the subject effects on each of the feeding indexes, SAS Procedure General Linear Model (PROC GLM) and SAS PROC Mixed Model Analysis (MIXED) were used to handle the repeated measurements and random individual rat effect. ANOVA was done by SAS PROC GLM to especially study the individual rat effect, and the mixed-effect model analysis was done by SAS PROC MIXED to study trends over time treating individual rat effects as random effects. Correlation coefficients were applied to changes in FI, MN, and MZ in the period from day –3 to day 0. Significance is indicated in figures and text. Figures 2 and 3 include average values of feeding indexes along with their standard errors.

RESULTS

Period From Day –3 to Day 0

Because by definition the day of anorexia occurrence (day 0) is determined in hindsight (i.e., 3 days after there has been a consistent decrease in daily FI), this day is at variance with the actual biological day when anorexia has biochemically manifested. Based on visual observations, we have previously ascertained that
early cancer anorexia develops initially via a decrease in MN and then, sometime later, is accompanied by a decrease in MZ (1). Thus the data for FI, MN, and MZ were analyzed during day 2 to day 0 in an attempt to more clearly document this impression. In the period from day -3 to day 0 of anorexia, the average linear correlation coefficient was 0.095 between FI and MN in the control group, whereas it was 0.49 between FI and MZ (P < 0.01). This strongly indicates that the reduction of FI at the onset of anorexia is better explained by the change in MN, whereas the reduction in MZ occurs only later.

Pre-TNF Receptor Injection

As indicated in Figs. 2 and 3, there was no significant difference between mean feeding indexes of the study and control groups. As anticipated by the nature of randomization, there was no significant difference of the slopes between the groups.

Figures 2A and 3 show linear trends in the FI change and the body weight change, and the regression analysis done by SAS PROC MIXED suggested linear regression as a reasonable model to describe the changes in FI and body weight during this period.

Post-TNF Receptor Injection

An overview of Fig. 2 shows that a significant improvement in FI occurred after TNF inhibitor injection (Fig. 2A). This was a result of a significant improvement in MN (Fig. 2B) and a smaller but significant improvement in MZ (Fig. 2C) in the study group. Significant time trends and group effects on FI, MN, MZ, and body weight occurred via rates of change and mean levels. The occasional rise in MN shown in Fig. 2B on day 3 for the control group and day 5 for the study group was mainly the result of missing values. The changes in FI, MN, MZ, and body weight were examined more closely and are summarized as follows:

Food intake. As shown in Fig. 2A, FI in the control group continued to decrease, whereas it significantly improved in TNF-inhibitor-treated rats. Although t-tests for mean difference between the groups showed statistical significance only from day 5 after the injection, further analysis using SAS PROC MIXED was done to incorporate nonzero correlation among repeatedly measured FI and individual rat effect. The variable “day” was used as a regression variable in SAS PROC MIXED to understand the time course of changes.
trends among repeated measurements. Treating the individual rat effect as a random effect and using autoregressive error with lag one, AR(1), the day effect, and the group \( \times \) day interaction effect were found to be significant with \( P < 0.001 \). The regression equations were estimated as \( 15.932 - 1.118 \times \text{day} \) for the control group and as \( 13.893 + 0.116 \times \text{day} \) for the study group. The linear coefficient for the control group, \(-1.118\), was significant \((P < 0.001)\), whereas the linear coefficient for the study group, \(0.116\), was not significant \((P = 0.300)\).

Several options for the covariance structure, such as AR(1), compound symmetry, and unstructured autocorrelation have been examined, and the AR(1) structure was determined to be the best one on the basis of Akaike's Information Criterion and Schwarz's Bayesian Criterion, as well as on the fact that it is natural for this kind of data to have correlations that are larger for nearby times than for far-apart times.

**Meal number.** The changes in mean daily MN during the postinjection period are shown in Fig. 2B and indicate a significant improvement in TNF-inhibitor-treated rats compared with controls. Because of a large within-group variability \((P = 0.001; \text{ANOVA})\) there was no significant mean difference between the groups on a daily basis. ANOVA done by SAS PROC GLM indicated a significant within-group variability \((P = 0.001)\). However, noting that the average daily meal number indicated a curvilinear trend and that the linear fit is reasonable only for half of the 16 rats in the study, a mixed-effect model with quadratic and linear trends was applied. The model with AR(1) covariance and the random rat effect was used treating day and day \( \times \) day as regression variables. The day \( \times \) day effect was significant \((P = 0.007)\), and the day effect had a \(P = 0.08\). No interaction term was found to be significant in the test for fixed effects, but the examination of individual regression equations below indicates interaction between day \( \times \) day and group.

The estimated regression equations for the model with the linear and the quadratic time trends were \(11.080 + 1.335 \times \text{day} - 0.258 \times \text{day} \times \text{day}\) for the control group and \(13.019 + 0.847 \times \text{day} - 0.155 \times \text{day} \times \text{day}\) for the study group, where the regression coefficients of \(1.335, 0.847, \text{and} \ -0.155\) are not significantly different from zero. The coefficient for the quadratic term for the control group, \(-0.258\), was significant \((P = 0.017)\). It implies that the daily mean meal number remains constant for the study group, whereas it increases with quadratic time trend in the control group.

**Meal size.** The changes in mean daily MZ during the postinjection period are presented in Fig. 2C and show a significant improvement in TNF-inhibitor-treated rats compared with controls.

Again, because of a large within-group variability \((P < 0.001; \text{ANOVA})\), there was no significant mean difference between the groups. Treating day as a regression variable, a mixed-effect model with quadratic and linear time trends was applied, and there was no indication of a significant quadratic effect. For a model with only the linear time trend and interaction effect, SAS PROC MIXED indicated day and day \( \times \) group as significant effects \((P < 0.0001\) and 0.041, respectively).

The estimated regression equations are \(1.165 - 0.068 \times \text{day}\) for the control group and \(1.090 - 0.023 \times \text{day}\) for the study group, where the linear coefficient of \(-0.023\) for the study group was not significantly different from zero. The coefficient for the linear term for the control group, \(-0.068\), was significant \((P < 0.001)\). The results from the mixed-effect model analysis imply that the daily mean MZ remains constant for the study group, but it decreases with a linear time trend in the control group. Although Fig. 2C indicates a curvilinear pattern for the control group, the nonlinear terms were not significant mainly because of a large rat-to-rat variability.

**Body weight.** Figure 3 shows the change in body weight during the postinjection period and indicates a significant improvement in TNF-inhibitor-treated rats compared with controls. As outlined for the MN and MZ, there was no significant mean difference between the groups \((P < 0.001; \text{ANOVA})\).

Body weight decreased over time in the control group, whereas it increased in the study group. The mean average increase rate of \(1.48 \pm 0.71\ \text{g/day}\) showed a significant linear time trend \((P = 0.050)\) and a moderately significant interaction effect \((day \times day \times group; P = 0.078)\). The estimated regression equations are \(351.634 + 4.683 \times \text{day} - 0.582 \times \text{day} \times \text{day}\) for the control group and \(351.788 + 1.048 \times \text{day} + 0.040 \times \text{day} \times \text{day}\) for the study group, where coefficients for the linear and quadratic terms for the control group, \(4.683\) and \(-0.582\), were significant \((P = 0.029\) and 0.025, respectively). The regression coefficients of \(1.048\) and \(0.040\) for the study group were not significant for this model because of overfitting. When a linear time trend model was fitted for the study group rats (see Fig. 3), the regression equation was estimated as \(351.289 + 1.372 \times \text{day}\) and the linear coefficient was highly significant \((P = 0.002)\).

**DISCUSSION**

The findings from this experiment in male Fischer 344 rats show 1) that during the onset of anorexia \((day - 3 \text{ to day } 0)\), FI decreases by an exclusive decrease in MN, joined only later by a decrease in MZ, suggesting an independent and temporally differential effect of the tumor; 2) that with the onset of anorexia \((\text{day } 0)\), in the controls there is a progressive decrease in FI via a decrease in MN and MZ, resulting in a decrease in body weight; and 3) that inhibition of TNF-\(\alpha\) activity by a soluble p55TNF receptor construct results in improved FI via both MN and MZ, leading to improved body weight.

The role of cytokines in the development of cancer anorexia has been repeatedly shown in experimental animal models \((11, 20)\). Cytokines initiate a cascade of events that ultimately leads to a state of wasting, malnourishment, and eventually death. The improvement of FI \((19)\), the normalization of metabolic changes...
(10), and the restoration of body weight (19) that has been reported after tumor removal support this hypothesis. TNF-α is involved in the anorexia associated with tumor growth, as suggested by the use of anti-TNF antibodies in anorectic tumor-bearing rats, resulting in enhanced food intake (28).

In our study, after the injection of the TNF inhibitor in anorectic tumor-bearing rats, we observed a significant improvement in FI as a result of improvements in both MN and MZ. The effects of TNF-α and TNF inhibition on the feeding indexes of MN and MZ have been previously investigated (30, 31), showing a main effect on MN. Our present data confirm and extend these reports by showing that TNF inhibition during tumor growth improves MZ as well as MN. The apparent difference between our results and those previously reported is likely a result of the different settings of the studies; i.e., we used tumor-bearing rats in which the anorexigenic stimulus of TNF-α is chronically exerted, whereas previous studies tested normal rats that acutely received pathophysiological concentrations of TNF-α.

A peculiar feature of the beneficial effect of the dimeric, pegylated 55-kDa TNF receptor construct on the anorexia of cancer is its delayed occurrence. In our study, tumor-bearing rats treated with the TNF-α inhibitor gradually improved FI. A likely explanation for this phenomenon might lie in the interaction existing between TNF-α and its receptor on target organs. At the onset of anorexia, it might be assumed that most of the TNF receptors are bound with the soluble TNF-α. Our pegylated construct does not have any effect on the complex TNF-α/TNF receptor, but it reduces the amount of soluble TNF-α available for binding. It is therefore conceivable that when new TNF receptors are made available on the cell surface or within a group of neurons, according to their turnover rate, fewer TNF-α molecules can bind to them. This in turn might cause the delayed improvement of anorexia observed in our rats.

In our experiment, study rats also showed a significant improvement in body weight compared with control rats. This is subsequent to the improved FI and may also reflect the role of TNF-α in tumor-induced acceleration of skeletal protein net degradation and reduction of muscle protein synthesis (17). TNF-α also acts as a tumor growth factor (6) by promoting angiogenesis (21); study rats showed a reduction of the mean tumor size, although nonsignificant; by the end of this relatively short study. Further studies using a different protocol for administration of the TNF receptor construct may clarify this point.

This study provides a further step in the understanding of the pathogenesis of a multifactorial syndrome such as cancer anorexia, gives further insight into the comprehension of the mechanisms that regulate feeding activity, and helps to better elucidate the role of the p55 TNF receptor. Whether the soluble pegylated 55-kDa TNF receptor-construct can be considered as a potential future drug to ameliorate anorexia in cancer patients remains to be evaluated.

Perspectives

The target organs for TNF-α in regulating feeding activity still need to be better characterized. Thus our present data cannot be related to any specific effect on a selective site. However, the hypothalamus plays a major role in the regulation of FI and contains TNF-sensitive neurons located in different areas, including the VMN and LHA (8, 24). It is therefore likely that the observed effects might be, at least in part, mediated by these hypothalamic sites. Also, the enhancing effect of the TNF inhibitor on MN and MZ in anorectic tumor-bearing rats is consistent with the hypothesis that the VMN and LHA influence these feeding indexes (16). It is now recognized that the brain is provided with a redundant series of neuroimmunoendocrine systems to maintain the homeostasis of FI, i.e., the close and inverse relationship between MN and MZ (35). These systems appear to involve a number of anatomically and functionally related brain areas and nuclei, including the VMN and the LHA. In the past, we showed 1) that the size of a meal is related to LHA-dopamine concentrations (16) and 2) that the onset of cancer anorexia is associated with low dopamine and high serotonin levels in the VMN (1). Furthermore, the injection of a serotonin receptor blocker into the VMN at the onset of cancer anorexia reverses the reduced FI by an exclusive increase in MN (12). These findings, together with the observation that reduced FI at the onset of cancer anorexia is brought about by reduced MN, strongly suggest an early influence of tumor associated cytokines on the VMN of the hypothalamus, thus accounting for the reduction in MN, which is then followed by the involvement of the LHA and the reduction in MZ.

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