Cytokine and Cyclooxygenase-2 Protein in Brain Areas of Tumor-bearing Mice with Prostanoid-related Anorexia

Wenhua Wang, Christina Lönroth, Elisabeth Svanberg, and Kent Lundholm

Surgical Metabolic Research Laboratory, Landberg Laboratory for Cancer Research, Department of Surgery, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden

ABSTRACT

Evidence suggests that cytokines in the central nervous system are mediators behind anorexia in tumor-bearing hosts. We have therefore evaluated, by immunohistochemical image analyses, time course changes of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF) α, IL-6 receptor (gp130), IL-1 receptor I, and cyclooxygenase (Cox)-2 protein in brain cortex, hippocampus and the ventromedial hypothalamic nucleus (VMH) in tumor-bearing mice with prostanoid-related anorexia. Pair-fed non-tumor-bearing mice were used as controls. Prostaglandin E_2 (PGE_2) was provided systemically to freely fed, non-tumor-bearing mice to confirm a role for prostanoids in modulation of brain cytokines and food intake.

Time course changes of IL-1β were significantly different between tumor-bearing mice and pair-fed controls in the hippocampus but not in the VMH. TNF-α in the hippocampus and VMH did not show any significant difference between tumor-bearing mice and pair-fed controls, whereas TNF-α showed a small increase over time in brain VMH. IL-6 content did not show any significant alterations among tumor-bearing and pair-fed mice but increased significantly over time in both the study and control group. Cox-2 in brain hippocampus and VMH showed a statistically significant rise in both tumor-bearing and pair-fed controls, with no difference between animal groups. Systemic provision of exogenous PGE_2 to non-tumor-bearing mice altered brain cytokines significantly in the hippocampus and VMH with associated changes in food intake. Our results demonstrate that some differences (IL-1β) occurred in brain cytokines comparing tumor-bearing and pair-fed, non-tumor-bearing mice but within unexpected decreased levels in brain tissue from tumor-bearing mice. Surprisingly, many time course changes in brain cytokines were similarly altered in tumor-bearing and pair-fed mice. Our observations do not support that up-regulation of brain cytokines explains or promotes anorexia in cancer disease. Rather, cytokine and Cox-dependent alterations in brain tissue seemed to be secondary to a decline in food intake and related to subsequent stress hormone activities.

INTRODUCTION

Anorexia markedly worsens overall patient survival and has been implied as a major factor in the ultimate demise for individuals in up to 50% of cancer patients (1, 2). Although peripherally produced cytokines, particularly IL-1β, TNF-α, and IL-6, are mediators of cachexia (3–12), there is circumstantial evidence that brain cytokines are also important together with neurotransmitters and neurotrophic factors in mediating anorexia in tumor disease. IL-1β has been reported to induce anorexia after intracerebroventricular injections (13–16), and a positive correlation between food intake and IL-1α concentrations in cerebrospinal fluid was observed in anorectic tumor-bearing rats (17), whereas intra-VMH injections of an IL-1 receptor antagonist improved food intake (17). Accordingly, up-regulation of IL-1β mRNA in brain tissue may be a significant factor behind anorexia in tumor-bearing rats (18). Unlike IL-1β, TNF-α and IL-6 had only short-term anorectic effects when injected intracerebroventricularly, and tolerance was developed after repeated injections (19, 20). Anorectic effects of IL-1 may be mediated by prostaglandins based on the observation that pretreatment with ibuprofen completely blocked anorectic effects of IL-1 (21). Cox-2, an inducible Cox, exists extensively in brain tissue and was up-regulated in rodent brain in response to systemic administration of bacterial lipopolysaccharide (22, 23). Thus, the expression of cytokines and corresponding receptors in relationship to Cox-2 expression in various brain areas may be important for the development and progression of tumor-induced anorexia. Therefore, the present study has evaluated time course changes of brain cytokines and Cox-2 in a tumor model with cytokine- and prostanoid-dependent anorexia.

MATERIALS AND METHODS

Experimental Procedures. Adult, female age-matched C57BL/6 mice (22–27 g; Bomholtgård, Denmark) housed in groups of four to five in plastic cages, received laboratory rodent chow (ALAB AB; Stockholm, Sweden) and tap water ad libitum 14 days prior to experimentation in a temperature-controlled room with a 12-h dark/light cycle. The animals were placed on a wire floor to adapt to the experimental conditions 3 days prior to experimentation.

Tumor-bearing Mice. Sixty-three mice were divided into four groups with tumor-bearing (n = 21), pair-fed non-tumor-bearing controls (n = 21), freely fed healthy non-tumor-bearing controls (n = 10), and baseline controls (n = 11). With light anesthesia (Ketalar, Rompun i.p.), mice were implanted s.c. bilaterally in the flanks on day 0 with 3–5 mm^3 of a transplantable methylcholanthrene-induced sarcoma (MCG101). Pair-fed control mice underwent a sham operation and were pair-fed to match the food intake in tumor-bearing mice with free access to tap water as described (24). Freely fed healthy control mice were housed under similar conditions as tumor-bearing mice but were given free access to laboratory rodent chow and tap water. Daily food intake and body weight were registered between 8:00 a.m. and 9:00 a.m. Eleven mice in the baseline control group were killed 3 days later after being placed on a wire floor to determine day 0 values of cytokines, gp130, IL-1RI, and Cox-2 in immunohistochemical image analyses. Tumor-bearing and pair-fed mice were sacrificed in subgroups of seven on days 4, 7, and 14. Blood samples were obtained by cardiac puncture after anesthesia. The brains were immediately fixed in situ by perfusion. The vascular bed was rinsed by 20 ml of saline through the left ventricle of the heart, followed by perfusion with 20 ml of 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were rapidly removed and fixed in buffered 4% paraformaldehyde at room temperature for 20–24 h after perfusion and in situ fixation. The samples were paraffin embedded and cut into 8-μm sections for immunohistochemical staining analysis. The sections were allocated between interaural line 2.46 and 1.86 mm, bregma −1.34 and −1.94 mm, according to The Mouse Brain in Stereotaxic Coordinates (25). The VMH was verified by Wright hematoxylin staining under microscope. 

PGE_2. Provision to Non-Tumor-bearing Mice. Non-tumor-bearing mice received a micro-osmotic pump (ALZET 1007D) placed in the peritoneal cavity. The pump released PGE_2 (high dose, 1872 μg/100 μl, n = 13; or low dose, 312 μg/100 μl, n = 4) or vehicle (n = 13) at a rate of 0.5 μl/h. Food intake and body weight were recorded daily between 8:00 a.m. and 9:00 a.m.
pg/ml throughout the experimental period in pair-fed controls (Fig. 2). Food intake and carcass weight started to decline significantly at day 7 in tumor-bearing mice (Fig. 3; P < 0.01). It was technically feasible to pair-feed non-tumor-bearing mice, corresponding to the food intake of tumor-bearing mice, which appeared to be 1 g/mouse/day less than consumed by freely fed non-tumor-bearing mice. Carcass weight in pair-fed mice did not decrease statistically significantly as confirmed for tumor-bearing animals (Fig. 3), as observed in our previous findings (24, 28–33).

Time course changes of IL-1β were significantly different between tumor-bearing mice and pair-fed controls in the hippocampus but not in VMH, whereas alterations in IL-1β over time were statistically significant in hippocampus and of borderline significance in VMH. Interaction between group and time for IL-1β was statistically significant in the hippocampus but not in VMH (Fig. 4). TNF-α in the brain hippocampus and VMH did not show any significant difference between tumor-bearing mice and pair-fed controls, whereas TNF-α alterations over time showed a significant increase in VMH (Fig. 5). IL-6 content in brain hippocampus and VMH was not different between tumor-bearing mice and pair-fed controls, but there seemed to be an increase over time in VMH, at least in tumor-bearing mice (Fig. 6).

Evaluation of brain VMH expression of gp130 (IL-6 receptor α) and IL-1RI is presented in Fig. 7. There were no significant differences between tumor-bearing mice and pair-fed controls for gp130 and IL-1RI in any observed aspects. By contrast, Cox-2 increased significantly over time in brain hippocampus and VMH of both tumor-bearing and pair-fed mice, without any difference between the groups (Fig. 8).

i.p. Provision of PGE2 to Non-Tumor-Bearing Mice. Anesthesia and surgical implantation of an i.p. micro-osmotic pump for i.p. provision of PGE2 caused a pronounced decline of food intake during 1 day after the experimental procedures in normal mice (Fig. 9). However, the restoration of food intake was significantly more rapid in sham controls compared with restorations in the study groups receiving either a high- or a low-dose of PGE2. All of the differences in food intake over time were statistically significant (P < 0.01) as well as interaction between group and time (P < 0.01). Accordingly, body weight changes were statistically different between the study and the sham group, which was also confirmed for body weight alterations over time (P < 0.01) and interaction between group and time (P < 0.01; Fig. 9). Non-tumor-bearing mice, receiving a high PGE2 dose, were also used for the evaluation of time course changes in brain cytokine content (IL-1β and TNF-α) in the cortex, hippocampus, and VMH (Fig. 10). The results demonstrated that IL-1β showed a statistically significant change over time in the cortex and VMH with a trend to a difference between study and sham groups in VMH. TNF-α showed a significant difference in the cortex between the study and the sham group only, whereas the changes over time were significantly altered in both the hippocampus and VMH areas.

DISCUSSION

The understanding of the mechanism behind cancer cachexia has improved during recent years, in part related to the use of well-defined experimental models of tumor-bearing animals. Accumulating evidence supports the concept that both systemic and CNS-derived cytokines are mediators behind anorexia-cachexia (3–20). In addition to such observations, it has also been assumed that prostanooids are involved as secondary mediators to the more well-recognized peptides of cytokines and classical growth factors (21, 34, 35). However, it remains unclear whether cachexia is the main cause or the consequence of anorexia. Pair-feeding experiments
Fig. 1. Microphotographs of hematoxylin staining and of IL-1β, TNF-α, IL-6, gp130, IL-RI, and Cox-2 immunostaining compared with negative control in the VMH. StreptABComplex/AP was used as developing system, and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium was used as substrate. ×40.
related to food intake in tumor-bearing mice have actually revealed few tumor-specific alterations in host metabolism (24, 28, 31, 36–39). Therefore, it has been difficult, even in animal models, to evaluate to what extent anorexia actually represents an adaptation in food intake secondary to the remodeling of host compartments in response to tumor growth. This uncertainty is particularly relevant for the patient situation (40), but similar uncertainties are also valid in a majority of tumor models. However, in some animal models it has been reported that carcass wastage occurs independently of anorexia (41). Therefore, it cannot be entirely excluded that anorexia is an independent and primary alteration that explains cancer cachexia, at least during certain periods of tumor progression (42). Irrespective of what is primary or secondary alterations in appetite control, it is important to understand regulating mechanisms behind the CNS control of food intake. Therefore, we have evaluated time course changes in brain content of cytokines (IL-1β, IL-6, TNFα, IL-6Ra, IL-1RI, and Cox-2) in the hippocampus and the VMH nucleus from inbred C57Bl mice s.c. implanted with a tumor (MCG101) promoting cytokine- and prostanoid-related anorexia/cachexia. This tumor model has been extensively characterized metabolically, and tumor-host alterations are in several aspects similar to metabolic alterations observed in unselected weight-losing cancer patients (43). We have found that carcass weight loss in tumor-bearing mice is closely related to alterations in food intake, which were all attenuated by cyclooxygenase inhibition (26, 35, 44). Pair-feeding and pair-weighted experiments in non-tumor-bearing mice have demonstrated that a majority of observed tumor-host alterations could be mimicked by a primary decline in food intake (adaptations to starvation; Refs. 24 and 45), although some differences that may be tumor related were observed (36). For example, resting energy expenditure in our tumor-bearing mice showed a different diurnal rhythm compared with the metabolism in purely undernourished non-tumor-bearing mice (30). Also, a majority of tumor-related metabolic alterations could be provoked by i.p. or s.c. injection of bacterial antigens extracted from Corynebacterium parvum (46–48). We have also reported that several classic tumor-host related alterations are tightly coupled to well-recognized stress alteration in the pituitary/adrenal axes (33, 49, 50). For example, systemic inflammatory reactions in response to growth of the malignant tumor were highly dependent on the presence of the adrenals in mice (33, 50). Thus, our previous work clearly demonstrates that tumor-host metabolism can be secondary to a primary decline of food intake, partly mediated by cytokines, prostanoids, and classic stress hormones, which all introduced secondary alteration in energy metabolism and hormone sensitivities.

On the basis of the above-mentioned evidence, we have now evaluated to what extent brain cytokines of tumor-bearing mice are differently related to brain cytokines in pair-fed, non-tumor-bearing mice. The experimental approach is thus similar to our previous investigations on metabolic alterations in liver and peripheral tissues of MCG101-bearing mice (24). The results in the present study confirm our previous findings that anorexia becomes significant 6–7 days after tumor implantation, and that carcass weight probably starts to decline somewhat earlier than food intake of
MCG101-bearing mice (38). These repeatedly observed alterations suggest that the initial growth of a tumor can be sufficiently supported by redistribution of metabolic compounds (substrate and energy) within the host without introducing a strictly parallel attenuation of food intake. Thus, it is likely that anorexia becomes evident when the tumor compartment has reached a certain metabolic proportion of the entire host. Accordingly, it is still difficult to judge what the primary cause of anorexia is, tumor-related factors or being an adaptation to initial alteration in basal energy metabolism with stress hormone alterations. However, a clear cut difference between the tumor-host condition versus a pure state of underfeeding (pair-fed mice) is the observed increase in plasma PGE$_2$ of tumor-bearing mice being highly correlated to the tumor burden (Fig. 2). This fact underlines that a state of progressive undernutrition in tumor-bearing hosts is a mixed condition with both systemic inflammation and pure undernutrition.

Our present experiments were designed to evaluate time course changes in brain peptides when expressed as a relative change versus baseline levels in the brain of freely fed, well-adapted, non-tumor-bearing mice (day 0, 100%). However, quantification of immune-histochemical alterations are usually subjected to a comparatively high degree of variation, particularly when performed on different groups of animals. Also, intersample variations occur. Therefore, we have focused on a statistical model with evaluation of changes over the entire experimental period, with the primary emphasis on differences between study and control mice. This approach may decrease the possibility to identify or confirm small but statistically significant differences among the tumor and the control groups. On the other hand, our approach will also decrease the risk for overinterpretation of suggestive changes appearing by chance. To compensate for a comparatively low sensitivity in the statistical analyses by comparing repeated samples over a 2-week period among study and control mice, we have also indicated

![Fig. 4. Time course changes of IL-1β in the brain hippocampus and VMH of tumor-bearing mice and pair-fed controls (hippocampus: between groups, $P < 0.01$; over time, $P < 0.01$; interaction between group and time, $P < 0.01$; VMH: between groups, $P = 0.41$; over time, $P = 0.09$; interaction between group and time, $P = 0.29$; seven animals in each observation point). Bars, SE.](image)

![Fig. 5. Time course changes of TNF-α in brain hippocampus and VMH of tumor-bearing mice and pair-fed controls (hippocampus: between groups, $P = 0.65$; over time, $P = 0.73$; interaction between group and time, $P = 0.93$; VMH: between groups, $P = 0.44$; over time, $P < 0.01$; interaction between group and time, $P = 0.45$; seven animals in each observation point). Bars, SE.](image)
statistical trends as well as significant interactions between groupings of animals and the observation time during the experiments. Several interesting observations were made. IL-1β was the only cytokine with a statistically significant difference between tumor-bearing and pair-fed controls in any of the evaluated brain regions (hippocampus) but with unexpectedly low levels in tumor-bearing mice. TNF-α showed no difference between the groups, without any striking alterations over time. Also IL-6, which has earlier been identified as a significant cytokine behind alterations in peripheral tissue metabolism of tumor-bearing animals (27), did not show any difference between the groups, whereas there was a significant increase over time in VMH in at least tumor-bearing mice. Our findings suggest that brain cytokine metabolism seems to be more complex than the relationships reported in other tissues of tumor-bearing animals, where it is not unusual to observe a positive and proportionally increased relationship between elevated cytokine activity and altered tumor-host metabolism (51).

The lack of comparatively pronounced alterations in brain cytokines in the present study was probably not explained by differences at the corresponding receptor levels, because no changes were found in gp130 (IL-6 receptor α) and the IL-1RI. However, Cox-2 increased in the hippocampus of both tumor-bearing and pair-fed controls with a similar pattern in brain VMH. Thus, our results do not agree with conclusions based on observations published previously that IL-1 receptor antagonists improve food intake in anorectic tumor-bearing animals (17).

To further evaluate our previous proposals of a relationship between prostanoid activity and tumor-host metabolism, PGE₂ was provided by means of an i.p. micro-osmotic pump in non-tumor-bearing mice. These experiments confirmed that well-adapted mice are highly sensitive to stress reactions such as anesthesia and surgical manipulations, demonstrated by an initial decline to 10% of normal food intake in such animals (Fig. 9). However, food intake was most rapidly restored in sham injected controls compared with mice receiv-

Fig. 6. Time course changes of IL-6 in the brain hippocampus and VMH of tumor-bearing mice and pair-fed controls (hippocampus: between groups, $P = 0.72$; over time, $P = 0.86$; interaction between group and time, $P = 0.87$; VMH: between groups, $P = 0.44$; over time, $P = 0.01$; interaction between group and time, $P = 0.47$; seven animals in each observation point). Bars, SE.

Fig. 7. Time course changes of gp130 and IL-1RI in the brain VMH of tumor-bearing mice and pair-fed controls (gp130: between groups, $P = 0.56$; over time, $P = 0.30$; interaction between group and time, $P = 0.48$; IL-1RI: between groups, $P = 0.73$; over time, $P = 0.52$; interaction between group and time, $P = 0.37$; seven animals in each observation point). Bars, SE.
ing PGE₂. These experiments cannot represent or simulate a finely tuned physiological situation but reflect expected findings that systemic provision of PGE₂ can be translated into influences on food intake with subsequent changes in body weight and agree with the findings that alterations in feeding may be related to Cox-2 expression in the brain. Moreover, these short-term but consistent findings were associated with significant alterations in IL-1β and TNF-α in the cortex, hippocampus, and VMH (Fig. 10). Presently, the results from these simplified experiments with exogenous PGE₂ provision cannot be interpreted in a physiologically meaningful way but demonstrate a possible link between prostanoid metabolism outside the brain barrier and cytokine expression inside the CNS. Thus, cytokines may not only control production of prostanoids in general, but systemically derived prostanoids can perhaps also control or at least influence the brain cytokine production (52). It is also possible that changes in cytokine production outside the CNS can be translated into Cox-2 alterations inside the CNS (Fig. 8). Such a bidirectional interplay, across the blood brain barrier between prostanoids and cytokines, reflects the complex situation between metabolic cascades in tissues and CNS (53).

The overall impression of our present study is that few differences occurred in brain cytokines in a tumor condition with host wasting compared with a condition with pure undernutrition. Thus, many directional changes in brain cytokines were similarly altered in tumor-bearing and pair-fed mice. These observations do not support that up-regulation of brain cytokines has a major role to elicit and promote anorexia in tumor-bearing mice. Rather, cytokine and Cox-dependent alterations seemed to be secondary to the decline in food intake and may represent adaptations to stress hormone secretion promoted by the partial starvation in tumor-bearing and pair-fed mice.

Fig. 8. Time course changes of Cox-2 in the brain hippocampus and VMH of tumor-bearing mice and pair-fed controls (hippocampus: between groups, \( P = 0.54 \); over time, \( P < 0.01 \); interaction between group and time, \( P = 0.34 \); VMH: between groups, \( P = 0.23 \); over time, \( P = 0.01 \); interaction between group and time, \( P = 0.08 \); seven animals in each observation point). Bars, SE.

Fig. 9. Food intake and body weight changes after i.p. PGE₂ provision by a micro-osmotic pump in freely fed mice (food intake: between groups, \( P < 0.01 \); over time, \( P < 0.01 \); interaction between group and time, \( P < 0.01 \); body weight: between groups, \( P = 0.08 \); over time, \( P < 0.01 \); interaction between group and time, \( P < 0.01 \)). Bars, SE. The high-dose provision of PGE₂ corresponded to 220–240 μg/mouse/day, and the low dose corresponded to 35–45 μg/mouse/day (four to seven observations in each point).
Fig. 10. Time course changes of IL-1β (left panel) and TNF-α (right panel) in the brain cortex, hippocampus, and VMH of PGE₂-provided mice. For IL-1β: (cortex: over time, \( P < 0.05 \); VMH: between groups, \( P < 0.07 \); over time, \( P < 0.01 \); interaction between group and time, \( P < 0.01 \)). For TNF-α: (cortex: between groups, \( P < 0.05 \); interaction between group and time, \( P < 0.05 \); hippocampus: over time, \( P < 0.01 \); interaction between group and time, \( P < 0.02 \); VMH: over time, \( P < 0.02 \); interaction between group and time, \( P < 0.06 \)). A minimum of three observations in each point were made. Bars, SE.
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