PHYSIOLOGICAL EFFECTS OF CNTF-INDUCED WASTING

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The authors have shown previously that in addition to its survival effects on neurons and glia, ciliary neurotrophic factor (CNTF) induced potent cachectic effects and acute phase proteins when present in the peripheral circulation at concentrations of $\leqslant$10 ng/ml. These effects did not depend upon the induction of other cytokine family members. Described here are the specific physiological effects which systemic administration of CNTF can induce in somatic tissue. Mice implanted with C6 glioma cells, genetically modified to secrete CNTF, exhibited rapid catabolism of adipose tissue and skeletal muscle, depressed steady-state levels of glucose and triglycerides, elevations in red blood cell content, gall bladder hypertrophy and thymic atrophy, with a disproportionate loss of $CD4^+/CD8^+$ T cells. This cachectic wasting resulted in death over a period of 7–10 days. Implantation of the parental C6 line, or C6 cells which express a non-secreted form of CNTF, did not result in overt effects over this time period. These findings have implications both for the biology of CNTF family members, and the therapeutic use of factors such as CNTF in vivo.

Ciliary neurotrophic factor (CNTF) is one member of a family of related cytokines which include interleukin 6, interleukin 11, granulocyte colony stimulating factor, leukaemia inhibitory factor and oncostatin M.[1–3] CNTF is a 23 kDa cytoplasmic protein found predominantly in glial cells, particularly Schwann cells of the peripheral nervous system; with expression beginning in the late embryonic period.[4] As the protein lacks a canonical signal sequence,[5] the means by which CNTF gains access to its extracellular targets remains a subject of controversy. CNTF has been shown to promote the survival of a variety of neuronal and non-neuronal cells,[6–9] and can slow motoneuron degeneration in the murine mutants *wobbler* and *pmn*.[10,11] CNTF has also been shown to promote the in-vitro differentiation of O-2A progenitors to type-2 astrocytes, and sympatho-adrenergic precursors to cholinergic neurons.[12,13] Disruption of the CNTF gene has been shown to result in a small reduction in motoneuron numbers in adult mice.[14]

CNTF mediates its effects through a tripartite receptor complex composed of a CNTF-binding alpha receptor (CNTFrα), gp130, and LIF receptor beta.[4,15–18] LIFr beta and gp130 are expressed in a wide variety of tissues,[16,18] whereas, expression of CNTFrα is more restricted, being confined primarily to the central nervous system.[4,19,20] CNTFrα is most closely related to IL-6rα,[20] and null mutants for this receptor exhibit severe motor impairments.[21]

At least two extra-neural sites of CNTFrα expression are known, one within the liver and the other in skeletal muscle.[4,19,22] This raises the possibility that CNTF may exert effects outside the nervous system proper. Because all other members of this family [interleukin 6 (IL-6) leukaemia inhibitory factor (LIF), IL-11, and oncostatin M[23]] exert a variety of effects on metabolic processes,[16,23–26] we decided to examine the physiological effects of systemic application of the neurokine, CNTF.

RESULTS

Animals were divided into four treatment groups, receiving either: cell dilution medium alone (CDM), the parental cell line (rat C6 glioma), C6 cells which produced but did not secrete CNTF (CNTF-ns) or, C6 cells which produced and secreted CNTF (CNTF-s). Animals received 1×10^6 viable C6 cells, or an equivalent volume of cell dilution medium into the peritoneal cavity and were observed for a period of 1 week, at which time they were killed, exsanguinated, and their organs processed for histological examination. This time point was chosen because it preceded

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substantial immune activation caused by the implantation of C6 cells. We have previously shown that implantation of 1×10^6 CNTF-s cells results in a sustained concentration of active CNTF in blood plasma of approximately 10 ng/ml.[27]

General metabolic effects of CNTF

The presence of CNTF induced dramatic losses of adipose tissue. An example is shown in Figure 1A, which shows two CD1 litter mates at 7 days following the implantation of CNTF expressing cells. The initial body weights of both animals were 30 ± 0.3 g. The skin of both animals has been removed in order to delineate the underlying tissues more clearly. As can be seen in the figure, animals which received CNTF secreting cells have undergone a virtual elimination of adipose tissue, as well as some loss of skeletal muscle. Subcutaneous and brown fat stores are also affected in these animals. As indicated in the figure, animals which received a sister cell line which does not secrete CNTF did not exhibit these effects. This finding prompted us to examine the effects of CNTF on intermediary metabolites and plasma electrolytes more closely.

As shown in Table 1, implantation of C6 cells did not significantly alter plasma electrolyte levels, or serum calcium. However there was a substantial reduction in the concentration of plasma glucose and serum triglycerides in CNTF-s treated animals, which significantly exceeded the reduction observed in the CNTF-ns/C6 groups. Although the values shown in the table were collected at 7 days post-implantation, a similar CNTF-induced reduction in blood glucose apparent 2 days post-implantation (data not shown).

In order to rule out hepatic damage or the presence of hepato-biliary disease as a contributing factor in the depression of these intermediary metabolites, we assessed serum alanine aminotransferase (i.e. alanine transaminase, ALT) and serum alkaline phosphatase (sAP) levels. Alanine aminotransferase is present in hepatocyte cytoplasm and thus serum levels of this enzyme are one measure of hepatic lysis. Serum alkaline phosphatase is largely a reflection of the activity of the liver and bone isoenzymes, and thus is also useful in assessing certain forms of liver injury and bone marrow abnormalities. As shown in Table 1, ALT and sAP levels did not differ significantly between groups, being in the range of 42–50 U/l and 50–80 U/l, respectively. Typically, hepatic damage (and/or metabolic wasting) would also be expected to alter serum albumin levels. However, as determined from electrophoretic separations of serum proteins shown in Figure 1B, CNTF-s-treated animals do not undergo a change in total serum albumin 7 days post-implantation compared to other treatment groups.

The above findings are all consistent with an absence of hepato-biliary injury and/or obstruction. The reduction in serum triglycerides observed in CNTF-s-treated animals also supports this conclusion, because serum triglycerides normally rise under conditions of liver injury; and CNTF-s-treated animals did not appear jaundiced at the time of sacrifice. Thus the observed metabolic disturbances induced by CNTF do not appear to be due to direct injury to the hepato-biliary system. Instead, CNTF-s-treated animals exhibited an increase in proteins in the pre-beta (lipoprotein) region (Fig. 1B), which are known to be increased during acute phase reactions, consistent with previous observations.[27]

In some respects, the effects observed in the presence of CNTF resemble that of certain hyperthyroid states. In order to assess this possibility, serum thyroxine levels were determined for animals in each of the treatment groups. No significant fluctuations in these activities were observed in any of the treatment groups [total serum thyroxine $17.0\ \mu g/l \pm 0.34$, (SD)]. As described above, alterations in the level of serum albumin were not observed in any of the treatment groups. These data argue against perturbation of thyroid function as a primary cause of the observed wasting. In addition, as shown in Table 1, no significant alterations in serum calcium levels were observed in any of the treatment groups, arguing against a perturbation of parathyroid function (see bone marrow below).

Thus the presence of active CNTF in blood plasma appears to induce catabolic events in which the body's energy supplies are progressively depleted; ultimately resulting in a reduction of important energy intermediates such as glucose and triglycerides. These effects are beyond those induced by C6 cells alone. Consistent with this, we observe a more rapid depletion of liver glycogen in CNTF-s, as compared to CNTF-ns treated animals during post-implantation days 1–3 (data not shown). Morphometric and histological examination of the internal organs of CNTF-s-treated animals showed remarkably few additional anomalies, and did not exhibit the generalized wasting observed in adipose and skeletal muscle. However, these animals did exhibit several tissue-specific changes as described below.

Thymic effects of CNTF

In mice implanted with CNTF-secreting cells, but not C6 or CNTF-ns cells, an acute thymic atrophy was noted, as shown in Figure 2A. Thymic volumes were found to be decreased an average of $40\% \pm 5.4$ (SD) for CNTF-s-treated animals compared to the CDM group ($n = 20$ animals/group). This atrophy was not the result of generalized tissue wasting, because glands such as the submaxillary showed no

TABLE 1. Serum metabolites (M), electrolytes (E) and enzymes

M/E (mmol/l)	CDM	C6	CNTF-ns	CNTF-s
glucose	10.5 ± 0.22	9.0 ± 0.60	9.2 ± 0.43	6.6 ± 0.33* **
triglycerides	1.20 ± 0.07	0.70 ± 0.03	0.72 ± 0.03	0.48 ± 0.03* **
calcium	2.57 ± 0.03	2.55 ± 0.02	2.59 ± 0.05	2.70 ± 0.04
sodium	150 ± 1.2	148 ± 1.1	149 ± 1.2	152 ± 1.2
potassium	8.6 ± 0.3	9.1 ± 0.2	8.9 ± 0.4	8.9 ± 0.5
chloride	112 ± 1.0	110 ± 1.1	109 ± 0.8	110 ± 1.0
Enzymes (units/l)				
Alanine transaminase	42 ± 3	50 ± 5	46 ± 4	47 ± 5
Alkaline phosphatase	65 ± 4	53 ± 6	75 ± 5	60 ± 7

Triglycerides = total serum triglycerides. Values were obtained at 7 days post-implantation. Values are given as the mean ± SEM. $n \geqslant 6$ animals per group. *$P < 0.05$ mock vs CNTF-s, **$P < 0.05$ CNTF-ns vs CNTF-s.

overt changes in CNTF-s treated animals. An analysis of the thymocyte populations by flow cytometry using CD4 and CD8 markers is shown in Table 2. Animals receiving CNTF-s exhibited a five-fold reduction total thymocyte number compared to the CDM or CNTF-ns groups. Animals injected with the parental C6 cells had values similar to those observed for the CNTF-ns group (data not shown). CNTF-s-treated animals exhibit a particularly large depletion of CD4/CD8 double positive thymocytes and showed a concomitant increase in their ratio of CD4/CD8 double negative cells. The proportion of $CD4^+$ and $CD8^+$ cells remains relatively normal.

During maturation, thymocytes proceed from a $CD4^-/CD8^-$ stage, to a $CD4^+/CD8^+$ stage; eventually maturing into CD4 or CD8 positive T cells. Taking into account the relative proportions of the remaining thymocytes in CNTF-s mice, CDM-treated mice exhibit a relative ratio of 25:322:13:75 ($4^-/8^-$:$4^+/8^+$:4^+:8^+), vs 33:26:4:25 for CNTF-s treated mice. CDM and CNTF-ns mice exhibit relatively constant numbers of $CD4^-/CD8^-$ thymocytes, with the primary depletion (92%) in CNTF-s-treated animals occurring in the $CD4^+/CD8^+$ population. Thus, it appears that the presence of CNTF does not interfere with early thymocyte maturation, but instead primarily depletes the $CD4^+/CD8^+$ population. This depletion of $CD4^+/CD8^+$ cells in turn diminishes the population which can give rise to $CD4^+$ (helper) or $CD8^+$ (suppressor) T cells. The result is that both of these populations are reduced to an equivalent extent (30% of that observed for CDM treated animals). The relative survival of $CD4^+$ and $CD8^+$ T cells (30%), over $CD4^+/CD8^+$ thymocytes (8%) in comparison to the CDM values, suggests that the maturing $CD4^+/CD8^+$ thymocytes are more sensitive to the effects of CNTF than are $CD4^+$ or $CD8^+$ T cells. This is consistent with previous observations which demonstrate that $CD4^+/CD8^+$ thymocytes are more sensitive to injury by a variety of cellular stimuli (glucocorticoids, dexamethasone, etc.), than are the other thymocyte populations shown in Table 2.[28,29] In addition, as shown in Table 2, total leukocyte counts of whole blood were not observed to vary significantly between CDM, CNTF-ns and CNTF-s-treated animals; again suggesting that these cells are not overtly sensitive to the effects of CNTF.

Haematological effects of CNTF

In animals which received CNTF-s producing cells, but not CNTF-ns or C6 cells, there was a clear increase in the amount of red pulp observed in bone marrow. An example of this shown for CNTF-ns and CNTF-s treated animals in Figure 2B (left panels). Haematoxylin/eosin stained longitudinal sections through the marrow capsule of femurs from CNTF-ns

TABLE 2. Percent distribution (%) of thymocytes in CNTF-treated animals

Marker	CDM	CNTF-ns	CNTF-s
$CD4^+$	3.1 ± 0.71	5.5 ± 0.62	4.6 ± 1.2
$CD8^+$	17.6 ± 4.1	30.0 ± 3.1	26.9 ± 3.9
$CD4^+/8^+$	73.5 ± 2.6	57.4 ± 5.5	30.1 ± 5.2* **
$CD4^-/8^-$	5.7 ± 1.8	7.0 ± 4.9	38.4 ± 5.0* **
Relative thymocyte number	100 ± 1.9	96 ± 1.8	20.0 ± 0.98* **
WBC's (1E9/L)	1.7 ± 0.2	2.4 ± 0.3	2.0 ± 0.4

Each animal received 1.0×10^6 viable cells (i.p.). Thymocytes were analysed 7 days post-implantation. Values are given as the mean ± SEM. $n \geqslant 4$ animals per group. *$P < 0.05$ mock vs. CNTF-s, **$P < 0.05$ CNTF-ns vs CNTF-s, FACS analysis, 10 000 thymocytes counted.

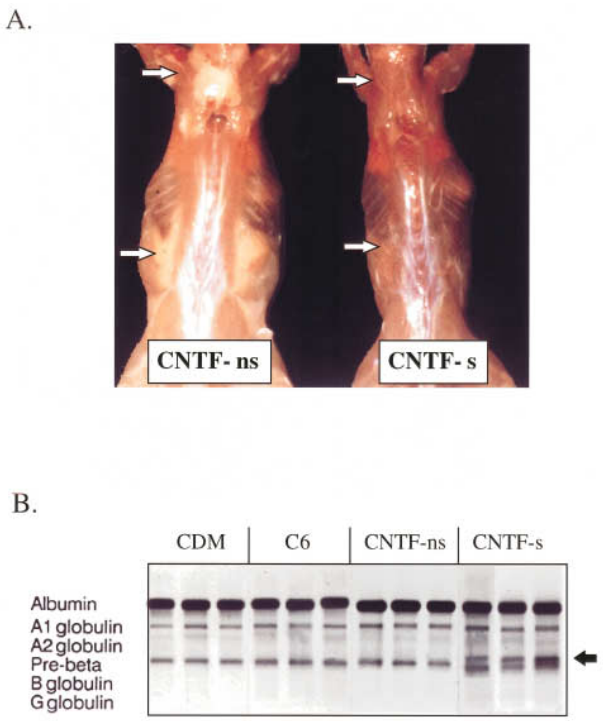

Figure 1. Systemic effects of CNTF

(A) Nine-week-old CD1 littermates at 1 week following injection of 1.0×10^6 CNTF-expressing cells. Skins have been removed to show underlying structures. Both animals had pre-implantation body weights of 30 ± 0.2 g. The animal on the left received C6 cells which produce but do not secrete CNTF, while the animal on the right received C6 cells which secrete CNTF. Sites of normal adipose deposits are indicated by the arrowheads. (B) Electrophoresis of serum globulins. Dilutions representing 2 µl of murine serum were subjected to gel electrophoresis to isolate their major globulin fractions. Serum components elevated in CNTF-s-treated sera are indicated by the arrowhead. For each group, three sera samples are shown.

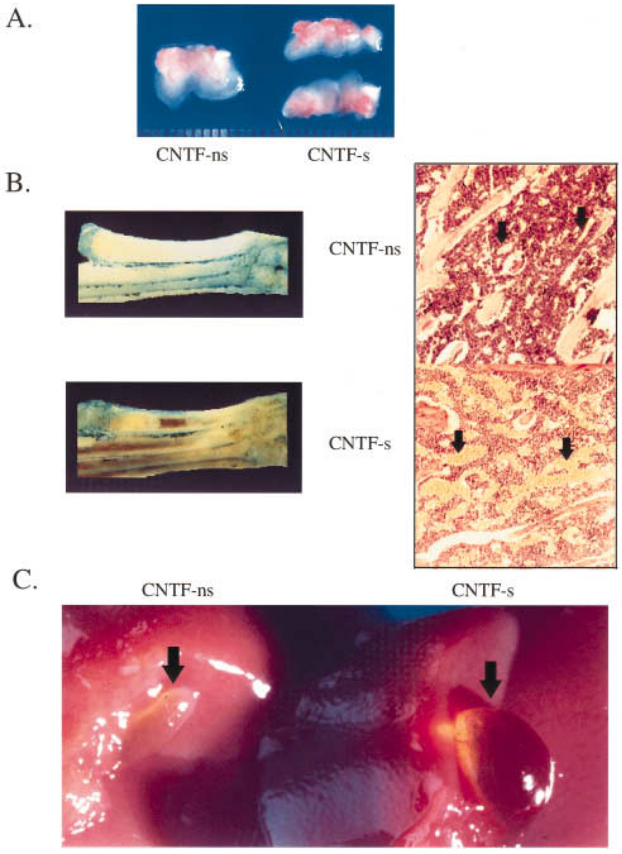

Figure 2. Effects of CNTF in specific tissues.

All tissues were collected at 7 days post-implantation, from animals receiving 1×10^6 viable cells. (A) CNTF-induced thymic atrophy. Thymuses from one CNTF-ns-treated, and two CNTF-s-treated animals are shown. Magnification $5 \times$. (B) Effects of CNTF on bone marrow. Left panels show exterior views of the metatarsals (cuniform bones oriented to the right) of a CNTF-ns and CNTF-s-treated animal following fixation. Panels on the right show haematoxylin/eosin stained sections of femur bone marrow in longitudinal section. Red blood cell islets are indicated by arrowheads. Magnification $85 \times$. (C) Effects of CNTF on gall bladder. Samples of liver CNTF-ns and CNTF-s treated livers are shown in which the ventral lobes have been removed in order to expose the gall bladder more clearly (arrowheads). Magnification $2.5 \times$ (reproduced here at 45%).

and CNTF-s-treated animals are also shown to the right. Arrowheads indicate the positions of several red blood cell (RBC) islets in these specimens. As can be seen in the figure, there is a substantial increase in the RBC volume occupied in the marrow cavity. This prompted us to examine the haematological profiles of these animals more closely.

As shown in Table 3, CNTF-s-treated animals exhibit increases in RBC number, blood haemoglobin, and blood hematocrit of approximately 120, 113 and 121%, respectively compared to CDM controls. In contrast, C6 and CNTF-ns-treated animals exhibited reductions in these parameters. This may be due to the presence of ascites fluid, which accumulates following the intraperitoneal transplantation of the C6 cells. If so, this effect may artificially depress the true values of the CNTF-s group. However, urine osmolarity (a

TABLE 3. Analysis of blood and urine of CNTF-treated animals

	CDM	C6	CNTF-ns	CNTF-s
RBCs (1×10^{12}12/l)	8.5 ± 0.32	7.5 ± 0.30	7.8 ± 0.43	10.2 ± 0.30* **
Haematocrit (l/l)	0.45 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	0.51 ± 0.01* **
Haemoglobin (g/l)	140 ± 2.7	120 ± 5.0	118 ± 5.0	170 ± 4.5* **
urine osmolarity (mmol/kg)	343 ± 3.9	333 ± 1.5	330 ± 2.5	338 ± 3.0

RBC—red blood cells. Values are given as the mean $\pm$ SEM. *$P < 0.05$ mock vs CNTF-s, **$P < 0.05$ CNTF-ns vs CNTF-s. $n \geqslant 6$ animals per group.

general measure of fluid homeostasis) was not altered in any of the treatment groups.

The increase in erythroid cells observed within the marrow cavity of the long bones of CNTF-s-treated mice, raises the possibility that CNTF may be acting directly on erythroid precursors. To examine this, erythroid progenitors were examined in the presence of increasing concentrations of CNTF (0 to 20 ng/ml) by standard Erythroid burst forming unit (E-BFU) assay. While progenitor cells cultured in high concentrations of CNTF exhibited greater numbers of erythroid colonies, this increase was not statistically significant. In addition, previous results have demonstrated that LIF can induce alterations in bone resorption and deposition, and dramatically elevate the number of megakaryocytes present in spleen and bone marrow.[25,30] IL-6 is also known to promote the maturation of megakaryocytes.[31] To examine the effect of CNTF on these processes, single cell suspensions of femur marrow, as well as serial cross-sections of bone marrow and spleen were prepared. No significant differences in either megakaryocyte numbers or splenic medullary centers among any of the treatment groups was observed.

Biliary effects of CNTF

As shown in Figure 2C, CNTF-s-treated animals exhibit dramatically increased incidence of gall bladder enlargement (arrowheads). A specimen from a CNTF-ns-treated animal representing the typical gall bladder appearance is shown for comparison. As shown in Table 4, CNTF-ns treated animals also showed an increased incidence of gall bladder enlargement compared to the C6 treated animals; perhaps due to a small amount of CNTF release from these cells. Examination of the C6 vs CDM group also suggests that implantation of C6 cells is not without effect. However, the presence of CNTF clearly causes an increase in the incidence of gall bladder enlargement. Dissection of the affected gall bladders revealed a lack of gall stones or other obstructions to bile emptying. Serum alanine aminotransferase and alkaline phosphatase levels, as described above, also suggest a lack of acute hepato-biliary inflammation and/or bile duct obstruction.

TABLE 4. Biliary effects of CNTF

Degree of gall bladder enlargement	CDM	C6	CNTF-ns	CNTF-2
− − −	>95%	60%	0%	0%
+	<5%	30%	50%	0%
+ +	0%	10%	25%	5%
+ + +	0%	0%	25%	95%

− − − = gall bladder <1 mm, + = gall bladder >1 mm, + + = gall bladder >2 mm, + + + = gall bladder ⩾3 mm. $n \geqslant 20$ animals per group.

DISCUSSION

The results presented here help elucidate the mechanism of CNTF-induced tissue wasting. The primary effects of circulating CNTF was that it strongly induces a catabolic state which progressively drains metabolic energy reserves, leading to a loss of adipose tissue (Fig. 1) and death. These effects do not appear to be primarily anoretic in nature, as dissection of the intestinal tract and colon near the time of death indicated the presence of continued food processing. The sustained presence of CNTF ultimately results in depletion of both short-term (hepatic glycogen) and long-term (adipose) energy stores, depressing these to the point at which levels of intermediary metabolites, such as serum glucose and triglycerides, are affected. Given that liver hepatocytes and skeletal muscle represent the primary extra-neural sites of CNTF receptor expression,[4,19] and the central role which the liver plays in intermediary metabolism, it is felt that the most probable explanation for the observed metabolic effects are in terms of a direct action on hepatocyte function. This in-vivo interaction also induces an acute phase reaction, as demonstrated by the up regulation of acute phase proteins. Consistent with this, CNTF has previously been shown to induce the synthesis of acute phase proteins in vitro in isolated liver hepatocytes and hepatoma cell lines (see Table 5). The severe cachexia induced by CNTF does not result from IL-6, LIF, or tumour necrosis factor alpha.[27] However, each of these cytokine family members has been shown to promote some form of cachectic wasting.[24,25,32] CNTF does not appear to mediate directly its effects via the central nervous system.[27]

CNTF exhibits greatest amino acid similarity to LIF and shares significant homology with IL-6. These factors also share similarities in their receptors, the CNTF receptor being composed of CNTRα, gp 130, and LIF-rβ; the LIF receptor being composed of gp 130 and LIF-rβ; and the IL-6 receptor being composed of IL-6rα and a dimer of gp130.[15,17,18] Consistent with this, these factors share some of the biological effects observed in CNTF-s-treated animals, as described below.

Within the thymus, animals treated with CNTF exhibited normal numbers of $CD4^-/CD8^-$ thymocytes, but a severe depletion of $CD4^+/CD8^+$ thymocytes, resulting in equivalent reductions in the number of intra-thymic CD4 and CD8 positive cells. No decrease in leukocyte numbers in whole blood was observed in these animals over the 1-week post-implantation period. This suggests a focused elimination of maturing thymocytes, rather than a generalized destruction of T cells. As shown in Table 5, thymic atrophy has also been observed in animals receiving LIF or oncostatin M, although the populations affected have not been

TABLE 5. Comparison of neuropoietic/haematopoietic cytokines

Method of application:	CNTF	LIF	IL-6	Onco. M	IL-11
Transgenic or cell implant	+[27]	+[25,30]	+[43,44]	+[49]	
Recombinant protein	+[22,27,34,40]	+[3,41]	+[45,46]	+[3]	+[51,52]
Acute phase reaction					
in vitro (hep./HepG2)	+[3,22]	+[3,41]	+[26,41,46]	+[3,50]	+[51]
in vivo	+[27,43,40]		+[43,44,45]		+[52]
Body wasting	+[27,34]	+[25,30]	+[45,47]	no[49]	no[45]
Haematopoiesis	+*	+[30,42]	+[35,45,54]		+[45,52]
Megakaryocytes	unchanged*	+[30]	+[31,44,54]	+[49]	+[45,51,52]
Bone effects	none obs.*	+[25]	(only est.)[48]	+[49]	+[48]
Serum albumin	unchanged*	decreased[25]	decreased[26,46]		
Thymic effects	atrophy*	atrophy[25,30]	T cell growth[53]	atrophy[49]	none rep.
Blood glucose	decreased*,[34]		unchanged[34]	unchanged[49]	

+/− indicates the presence of, or an increase in, the indicated property; *this publication; hep.—primary liver hepatocytes; none rep.—none reported; only est.—only under estrogen-depleted conditions.

described. Given the sub-populations affected in CNTF-s-treated mice and their lack of functional CNTF receptors, the most probable mechanism of this thymocyte loss is as part of a generalized stress response induced by the observed metabolic changes. It has been demonstrated that a similar pattern of thymocyte loss can be induced by glucocorticoids, which are stimulated in response to physiological stress.[28,29] It is interesting to note that in addition to causing apoptosis in developing thymocytes, glucocorticoids can increase erythrocyte numbers, stimulate lipoprotein synthesis, and cause thinning of the skin. These agents also inhibit glucose uptake in tissues; with the exception of liver, heart, brain and erythrocytes.[33] Glucocorticoids can also penetrate the blood–brain barrier to stimulate changes in the CNS.[33] Thus, it is likely that agents such as these participate in the induction of some of the secondary effects observed in CNTF-s-treated animals. Consistent with this, animals treated with recombinant CNTF have been shown to undergo a rise in corticosterone levels.[34]

Glucocorticoids are also known to inhibit lipolysis, stimulate gluconeogenesis and appetite, and increase serum calcium concentrations; so it is unlikely that they are solely involved in the observed metabolic deregulation. Indeed, these agents are normally thought of as part of a system which protects against the effects of fasting. However, the inappropriate stimulation of these agents could well contribute to the metabolic effects observed in CNTF-s-treated animals. Agents such as mineralocorticoids and aldosterone are unlikely to be involved as CNTF-s treated do not show significant alterations in their levels of blood electrolytes. Other potent metabolic mediators such as thyroxine do not appear to be involved in this wasting process, as T4 levels are not altered in any of the experimental groups. Similarly, parathyroid function does not appear to be substantially altered, as serum calcium levels are not significantly altered.

One early effect of CNTF is to induce a depression in drinking response.[27] It is therefore important to ascertain to what extent the effects observed in CNTF-s-treated animals are related to dehydration, which might concentrate blood components and affect reported plasma and sera values. Analysis of urine osmolarity, blood sodium, potassium and chloride levels, as well as counts of total leukocyte number, suggest that blood, plasma and sera values are not altered substantially by dehydration in CNTF-s-treated animals compared to controls. Indeed, dehydration would tend to concentrate the haemodynamic volume artificially elevating metabolite concentrations, instead of giving the depressions we observe in animals treated with CNTF.

The wasting observed in CNTF-s-treated animals is, in some respects, unique compared to that induced by other cachectins, as it does not cause a reduction in serum albumin by seven days post-implantation (see Table 5). Densitometric scanning and chemical analysis of serum samples, such as those shown in Figure 1B, indicate that albumin concentrations were within $\pm 5\%$ for all treatment groups at 1 week post-implantation; a period sufficient to cause a $35 \pm 5\%$ reduction in body mass.[27]

As shown in Table 5, the haematopoietic cytokines exhibit a variety of effects on bone and bone marrow. With respect to effects on erythrocytes, IL-6 has been shown to promote the proliferation of erythrocyte progenitors.[35] While mice receiving LIF do not demonstrate elevations in blood haematocrit, these animals do show increased numbers of nucleated erythroid cells in the spleen.[30] In the CNTF-s-treated animals used in this study, an increase in the numbers of erythroid cells in the bone marrow was observed, as well as an elevation in blood haematocrit and haemoglobin levels. While these elevations are moderate, the fact that there is a detectable rise in these values over a 7-day period suggests that these effects may be

substantial under more prolonged treatment. The observed changes in erythroid character do not appear to be mediated by CNTF at the progenitor cell level, given its lack of effect in in-vitro E-BFU assays. Animals receiving CNTF secreting cells did not exhibit statistically significant increases in megakaryocyte number or bone remodeling over a 7-day period; effects previously observed with other haematopoietic cytokines (see Table 5).

Thus, despite markedly similar biological effects, CNTF and LIF also exhibit some differences in their biological activities. These differences are particularly intriguing given the similarity of their receptors. While the circulating concentration of CNTF in this study is similar to that reported previously for LIF,[25,30] these factors may, under certain conditions, exert different biological activities due to differences in biological half-life and/or ligand-receptor avidity; resulting in different degrees of receptor activation. Alternatively these factors, which induce the heterodimerization and phosphorylation of LIF-β and gp130 through Jak family kinases,[15] could stimulate different intrinsic patterns of tyrosine phosphorylation, in turn activating alternative downstream signal paths which possess both convergent and divergent elements. An examination of the pattern of receptor tyrosine phosphorylation induced through the binding of specific ligands will thus be an important future step in our understanding of the potential signal responses of these cytokine receptors.

With respect to the dramatic increase in gall bladder size observed in CNTF-s-treated animals vs controls, a number of factors could potentially give rise to these effects. The possible primary causes include; acute inflammation or blockage of the bile duct; decreased food processing and/or gall bladder emptying; or increased production of bile components. In the case of CNTF-s-treated animals, acute inflammation and/or bile duct obstruction does not appear to be a factor, based upon serum alanine aminotransferase, serum alkaline phosphatase, and histological examination of the gall bladder. Gallstones were not observed in any of the treatment groups. As indicated above, examination of the gastrointestinal tract demonstrated the continued presence of food processing and blood electrolytes were within the normal range, suggesting that these are not the cause of the increases observed in CNTF-s treated animals. We feel that the most probable cause of the observed increase in gall bladder size is due to an enhanced bile acid synthesis from liver hepatocytes and/or increased bilirubin production from the catabolism red blood cells. The suggestion of the enhanced catabolism of RBCs may appear to be contradictory to the effects observed in the vasculature and bone marrow. However, a moderate increase in the catabolism of these cells would increase the rate of haemoglobin destruction and thus of catabolic products such as bilirubin in the bile. The destruction of RBCs would also stimulate the synthesis of red blood cells in the bone marrow. Consistent with this, we observe some increased haemolysis in the sera of CNTF-s-treated animals compared to other treatment groups. Also, the bile fluid in CNTF-s-treated animals appears darker (yellow) compared to that obtained from CDM-treated animals. However, bile fluid from all animals with enlarged gall bladders was of the same colour, regardless of the treatment group. A thorough examination of the gall bladder in LIF and IL-6-treated animals has not been reported. It will be of interest to see what effects, if any, these factors exert on the biliary.

The above results demonstrate that in addition to its CNS effects, CNTF can exert a variety of extra-neural effects. In summary, we provide here a physiological and anatomical description of these effects, demonstrating that CNTF induces profound body wasting, changes in immune and haematopoietic character, and effects on gall bladder function. Given the restricted pattern of CNTF receptor expression compared to that of other cachectins,[4,36] analysis of this wasting model may be useful in determining the biology of metabolic wasting. The mechanism of these effects will be important, both in terms understanding the underlying biology of CNTF and related factors, as well as establishing the safest means of delivering this protein in various clinical applications.

MATERIALS AND METHODS

Animals

Experiments were performed using age matched, nine week old CD1 female mice (Jackson Laboratories, Bar Harbor, ME). Starting weights for the CD1 animals were 29 ± 2 g. Animals were given food and water *ad libitum*, weighed daily, and raised under identical conditions in the same room of our animal facility. All experimental protocols conformed to Mount Sinai/University of Toronto animal colony care guidelines.

CNTF-producing cells

Stable CNTF-producing rat C6 cells were generated, cultured and implanted as previously described.[27] Secretory and non-secretory CNTF constructs were identical, with the exception that the secretory construct contained an 80-bp fragment encoding the human growth hormone signal sequence, fused in-frame to the start of the CNTF cDNA. Cells were injected into the peritoneal cavity of each animal, at a concentration of 5.0×10^6 viable cells/ml.

Analysis of murine blood, serum and urine

At the time of sacrifice, blood was drawn from each animal via trans-cardial puncture under deep anaesthesia. Coagulation was inhibited where necessary through the addition of heparin and EDTA to final concentrations of 1 U/ml and 2 mM respectively. Leukocyte, erythrocyte and platelet counts, as well as blood haematocrit were determined using a Sysmex model SP-1 NE-8000 automated blood analyser. The system was calibrated for the analysis of murine cells, using murine standards which were counted on a haemocytometer. Haemoglobin content was determined by lysing cells in the presence of sodium lauryl sulfate and converting iron from the ferrous to ferric state, thus forming methaemoglobin. This product was then combined with potassium cyanide to produce cyanmethaemoglobin, which was measured at 540 nm.

Serum was collected from blood coagulated for 1–2 h at 25°C, and stored at −20°C until used. Reagents were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise noted. All samples were run in duplicate against a set of known standards. Sodium, potassium and chloride concentrations were determined by ion-selective electrode binding, using a Ektachem 700 chemistry analyser. Serum calcium concentrations were determined by chelation to arsenazo III, and the colorimetric complex assessed at 605 nm. Serum glucose concentrations were determined by enzymatic oxidation with glucose oxidase, forming glucuronic acid and hydrogen peroxide. Peroxide formation was then determined by reaction with 4-aminoantipyrine and *p*-hydroxybenzene to form a quinoneimine which was then assessed at 505 nm. Serum triglycerides were determined by sequential reaction with lipoprotein lipase, glycerol kinase and L-glycerol phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide, which was analysed as given above. Alanine transaminase activity was assessed by the conversion of alanine and α-ketoglutarate to pyruvate and glutamate, followed by reaction with 2,4-dinitrophenylhydrazine to form a coloured product which was analyzed at 500 nm. Serum alkaline phosphatase activity was determined by the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate, monitored at 405 nm. Serum albumin concentration was determined by electrophoresis (see below), and by bromocresol purple binding, monitoring complex formation at 600 nm. Urine osmolarity was measured using an Advanced Instruments micro-osmometer, model 3MO, by freezing point depression.

Tissue fixation and histology

When animals were killed, tissues were dissected, washed in phosphate-buffered saline (PBS) at 4°C, then fixed in a buffered solution of 4% paraformaldehyde at 4°C for 48 h. Tissue wet weights were obtained prior to fixing. Following fixation, tissues were processed into paraffin blocks. Sections were cut at 10 μm and stained with Erlich's haematoxylin and eosin according to standard procedures.[37] In addition, cytospin smears from femur marrow were prepared and stained using the May–Grunwald–Geimsa procedure,[37] in order to determine the number of megakaryocytes present. This staining procedure was also employed on serial spleen and femur cross-sections.

For analysis of the skeleton and bone matrix, animals were dissected as completely as possible, and the remaining carcasses placed in 95% ethanol at room temperature for 48 h. Samples were then exposed to several beetle species which removed the remaining soft tissues. Skeletons were then stained with alcian/alizarin described previously.[38]

All processed samples and paraffin blocks were given coded identification numbers so that sections derived from them could be analysed in a blinded manner.

BFU-E, granulocyte-macrophage colony-forming units (GM-CFU) assay

Bone marrow cells were flushed from the right and left femur capsules of nine week old CD1 female mice into a solution of Hank's balanced salt solution, pH 7.4 at 4°C. Cellular debris was then removed by allowing it to settle to the bottom. Approximately 2.0×10^5 cells were then dispersed per 3-cm dish and cultured in supplemented Iscove's media as previously described[39] for 3 days. Recombinant CNTF was then added to the desired concentration in each dish and the cells cultured for an additional 4 days. At this time, plates were scored for the number of erythroid colonies they possessed, based upon their content of haemoglobin containing cells.

FACS analysis

Mice from each group were killed and their thymuses quickly removed and minced on ice in a 50:50 solution of alpha modified Eagle's medium (MEM) and F12 supplemented with 2% fetal calf serum (Hyclone Laboratories, UT). Following the removal of cellular debris, thymocytes were sedimented, and each sample resuspended in fresh media and split into four equal parts at a final concentration of 1.0×10^6 cells/ml. For a given sample, each tube was then incubated with either: FITC-conjugated anti-CD4, Phycoerythrin conjugated anti-CD8, anti-CD4/anti-CD8 (Sigma Immunochemicals, St Louis, MO), or media alone for one hour in the dark at 4°C. Samples were then washed three times with media and resuspended in 100 mM PBS (pH 7.4) at 4°C to a final concentration of 1.0×10^6 cells/ml. Samples were then analysed on an EPIC Elite flow cytometer (Coulter Electronics, Hileah, FL).

Globulin electrophoresis

Dilutions of serum from each group were separated on Beckman Paragon SPE gels by electrophoresis at 150 V for 3 h. These gels were then stained with Coomassie blue and air dried. Subsequent densitometric scanning and integration of the peak areas obtained, allowed quantitative comparison of the major serum proteins.

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