

Transplantable glucagonomas derived from pluripotent rat islet tumor tissue cause severe anorexia and adipsia

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From pluripotent pancreatic rat islet tumor tissue we have previously reported the isolation of stable transplantable glucagonoma tumor phenotypes in rats characterized by acute onset of anorexia. We now report that these tumors also cause severe adipsia. Food and water intake is reduced by more than 95% and is immediately cured upon tumor removal. Four anorectic tumor lines were all characterized as glucagonomas with high levels of proglucagon mRNA, and of two tested both were associated with highly elevated plasma levels of glucagon as well as of Glp-1_(7-36amide) in the host rat. This fetal processing pattern of proglucagon may be indirectly linked to the anorectic phenotype, since we have now isolated a non-anorectic glucagonoma with similar levels of proglucagon mRNA. Lack of anorexia/adipsia in SV-40-T-antigen driven glucagonomas in transgenic mice with similar fetal processing as reported by others suggests that our tumors produce a novel anorectic substance. This factor ranges among the most potent of its kind as a peripheral mediator involved in appetite and thirst regulation.

In summary, the glucagonomas provide an interesting tool with which to study the nature of severe anorexia as well as adipsia, and the identification of the active substance(s) may provide novel therapeutics for the treatment of obesity-related disorders such as NIDDM.

Key words: alpha-cell tumor; drinking; feeding; glucagon processing; islet ontogeny; weight loss; NIDDM.

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INTRODUCTION

In vitro cultures derived from a liver metastasis of a transplantable insulinoma (MSL-cells) share properties with early endocrine pancreatic stem cells [1] which are characterized by the ability to express multiple hormone genes within single cells [2]. Clonal MSL cultures are thus highly heterogeneous *in vitro* where different combinations of hormones can be detected within individual cells. Upon successive *in vivo* passage of MSL cells we were able to derive highly stable and homogeneous insulinomas as well as glucagonomas from common clonal origin (reviewed in [3]). This process most likely mimics a second step in normal islet differentiation where the expression of each of the four classical islet hormones is restricted to particular cell phenotypes [4].

Insulinomas and hypoglycemia

The clone, MSL-G2, predominantly produces glucagon, islet amyloid polypeptide (IAPP), and cholecystokinin *in vitro* [1,5]. Despite the fact that this clone displayed a very low frequency of insulin-producing cells and that insulin mRNA was undetectable by Northern analysis, it formed hypoglycemic tumors when passaged *in vivo* [6]. Selective transplantation for hypoglycemia resulted in the derivation of a stable line of small-sized tumors with highly active insulin and IAPP genes [5]. Subclones of MSL-G2 carrying a single transfected genomic copy of the human insulin gene [7] behaved similarly *in vitro* with almost no detectable expression of insulin. After *in vivo* passage, insulinomas were formed which, in addition, co-activated the human insulin gene with the endogenous rat insulin genes and human insulin and C-peptide were thus co-released to the circulation [6]. The transfected human insulin gene served two important purposes: 1) to allow

studies of an exogenous islet B-cell marker during islet tumor B-cell differentiation [8,9]; and 2) to provide a unique clonal marker since its integration into the rat genome in a particular cell is inherited in all daughter cells of that clone. It was thus possible to verify by Southern blot analysis that non-insulin-producing *in vitro* cells and the subsequently formed insulinoma indeed were of common clonal origin [6].

Glucagonomas and anorexia

Occasionally the hypoglycemic tumors formed as above also caused a severe anorexia. We hypothesized that this was due to the presence of another dominating cell type in these mixed phenotype tumors. By selective transplantation for anorexia it was possible to dissociate and establish stable anorectic tumor lines (Fig. 1) with normoglycemia. This process was repeated with the human-insulin-gene transfected subclones and thus it was unambiguously shown that hypoglycemic as well as anorectic tumors could be derived from common clonal origin [10]. Four independently derived anorectic tumor lines were all characterized as glucagonomas with a high content of proglucagon mRNA [11] and with silent insulin and IAPP genes [5]. Moreover, anorectic tumor lines were associated with highly elevated plasma glucagon levels (3000-6000 pM) in tumor bearing rats [10]. The aim of the present study was 1) to study food and water intake in the anorectic phase, 2) to evaluate the relationship of proglucagon processing and anorexia, and 3) to discuss a possible relationship to the classical studies by Coleman [12], where a similar severe anorexia is observed in parabiosis experiments between two otherwise hyper-phagic mouse strains, the diabetic (*db/db*) and the obese (*ob/ob*) mouse.

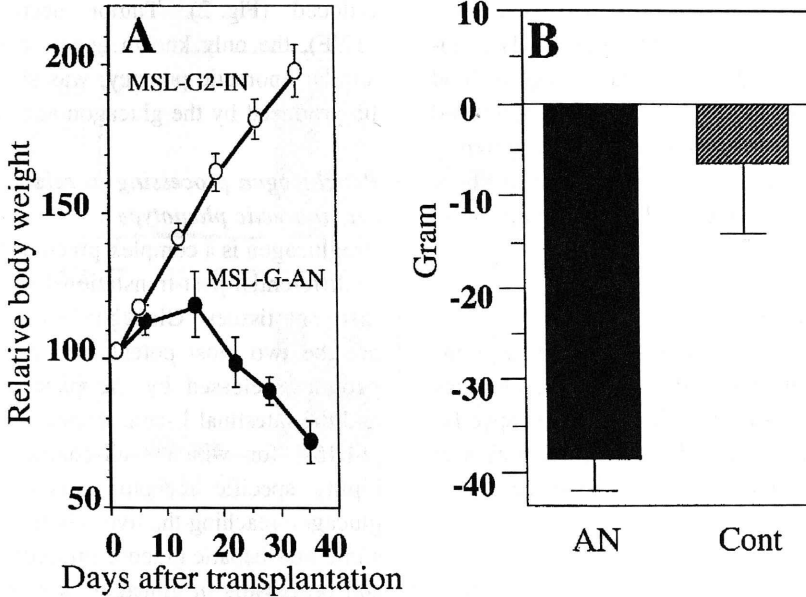


FIG. 1. A: Weight curves of rats carrying insulinomas (MSL-G2-IN, open symbol) or glucagonomas (MSL-G-AN, filled symbol), both derived from common clonal origin (adapted from (10)) ($n=4$ in each group). B: Accumulated weight loss (gram) per rat over a period of five days after transfer to metabolic cages. Glucagonoma bearing rats (black bar, left) rapidly lose weight after the acute onset of anorexia compared to control rats (hatched bar, right) ($n=6$ in each group). The apparent stress value of being transferred to metabolic caging is reflected by a minor weight loss also observed in the group of control rats (see Materials and Methods).

MATERIALS AND METHODS

Tumor transplantation

Fragmented tumor tissue ($< 1 \text{ mm}^3$) in RPMI 1640 with no supplements was injected s.c. in the back (in the shoulder/neck region) of NEDH rats in a volume of 20 - 30 μL , using a 1 mL syringe equipped with a 16 gauge needle. The tumor fragment was allowed to settle on top of the needle, and then gently forced into the needle prior to injection. By this method a very limited volume of fluid was co-injected with the tumor fragment. Blood glucose and body weight were measured once a week. The isolation of metastatic variants of a given transplantable tumor line was performed as follows: After onset of

anorexia the implanted subcutaneous tumor was excised from anesthetized animals. Such animals immediately resumed normal feeding behavior followed by rapid weight gain and were observed over a following period of up to six months. After sacrifice the liver or the lungs as the main target for possible metastatic spreads were examined carefully. If positive, the tumor was dissected and processed for further transplantation or for *in vitro* culture as described [1].

Measurements of food and water intake

At onset of anorexia tumor-bearing and control rats were transferred to metabolic cages, which allows the determination of food and water intake from preweighed containers as

well as of accumulated urine and faeces production during a 5-day test period. The animals were not adapted to the change in food texture (from regular pellets to powdered chow) prior to the transfer, which apparently caused some stress as evident from a slight weight loss also observed in non-tumor bearing control rats.

Proglucagon processing

Quantitation of proglucagon-derived fragments in plasma and tumor extracts was performed using various region-specific radioimmunoassays following gel filtration or HPLC separation as previously described [10,13].

RESULTS AND DISCUSSION

The anorexia caused by the glucagonomas was very severe and led to an almost complete stop in food intake, whereas control insulinomas of common clonal origin, reaching a similar size (0.5-1 g) during the experimental period, had no negative effect on weight gain (Fig. 1). The onset was almost acute and the observed weight loss was as high as 8-10 g/day/rat after entering the anorectic phase (Fig. 1). The rats appeared normally active in the initial anorectic period. Ultimately, they would die of cachexia unless the implanted tumor was removed. Upon tumor removal the feeding behavior was instantly normalized and characterized by a period of highly efficient weight regain [10]. When housed in metabolic cages the changes in feeding and drinking behavior were measured. Over a 5-day period after onset of anorexia the amount of consumed food was reduced by more than 95% in tumor-bearing rats compared to controls (Fig. 2). Surprisingly, also water intake was reduced to a similar extent (by approx. 90%). As a consequence, urine and faeces production was drastically

reduced (Fig. 2). Tumor necrosis factor (TNF), the only known agent so far with a similar anorectic potency, was shown not to be produced by the glucagonomas [10].

Proglucagon processing in relation to the anorectic phenotype

Proglucagon is a complex precursor subjected to differential post-translational processing in different tissues. Glucagon and Glp1_{7-36amide} are the two most potent biologically active peptides, released by the pancreatic α -cell and the intestinal L-cell, respectively (Fig. 3) [14,15], for which well-characterized and highly specific receptors exist. Pancreatic glucagon reaching the liver via the portal vein stimulates hepatic glucose production. The α -cell processing of glucagon is believed to be carried out mainly by the prohormone convertase, PC-2 [16]. Glp1_{7-36amide} is released from the intestinal L-cell following a meal and decreases blood glucose by potentiating glucose-induced insulin release from the pancreatic β -cell and by inhibiting glucagon secretion from the α -cell (for review see [17]). The pancreatic α -cell and the intestinal L-cell are thus highly specialized in their distinct and specific processing of the same proglucagon precursor. The association of anorexia and the glucagonoma phenotype was unexpected since experimental pancreatic glucagonomas in transgenic mice [18] with a pancreatic processing pattern [19] do not suffer from anorexia. However, severe weight loss is an integral part of the glucagonoma syndrome in man [20]. We therefore tested whether a particular pattern of proglucagon processing could be associated to the anorectic phenotype. In two anorectic lines tested, MSL-G-AN and NHI-5B-AN, the precursor was found to be processed extensively, leading to the excessive co-release of glucagon as well as Glp1_{7-36amide} (Fig. 3) [10]. A similar processing pattern has been reported during islet development [21]. Moreover,

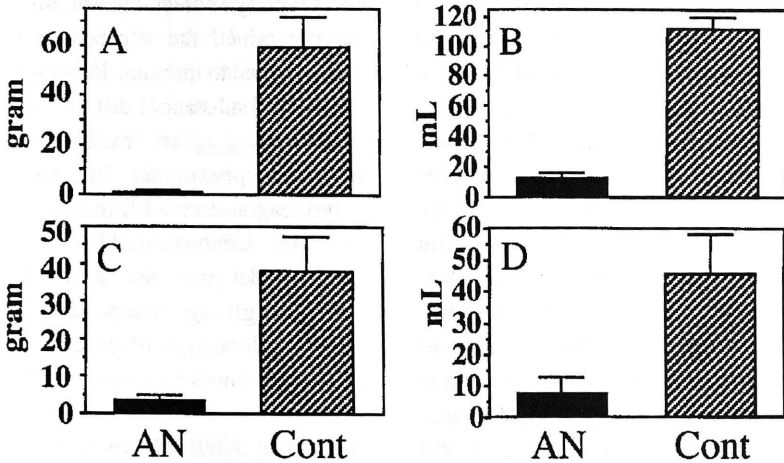


FIG. 2. Accumulated food (A) and water (B) intake over a period of five days is shown in the upper panels for groups of tumor-bearing and control rats (n=6 in each group). Lower panel shows faeces (C) and urine production (D) during this period. Black bars (AN): anorectic rats; hatched bars (Cont): control rats.

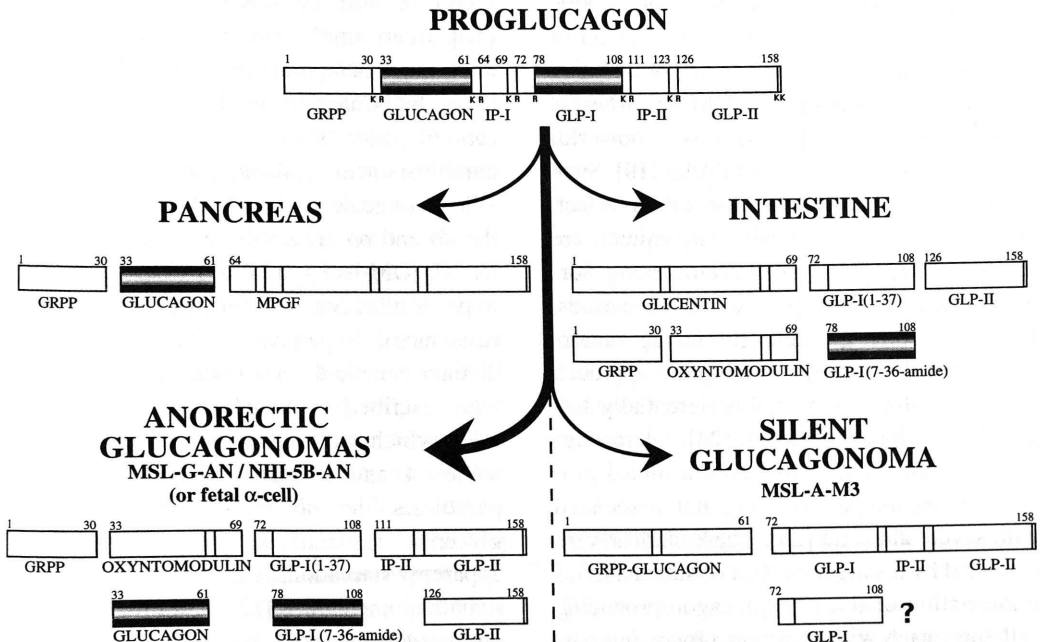


FIG. 3. Proglucagon processing in relation to anorexia. Biologically active glucagon and Glp-1 is visualized by shading in the various types of processing. The Glp-1/glucagon processing pattern was not determined (?) in the non-anorectic glucagonoma, MSL-A-M3. However, the major glucagon containing fragment with a free C-terminus was extended N-terminally (with the GRPP fragment).

we now report the establishment of another stable transplantable glucagonoma line, MSL-A-M3, which does not cause anorexia, but the type of processing remains to be clarified in detail. This tumor was derived from a liver metastasis of the anorectic line, MSL-A, and has by Northern analysis comparable levels of proglucagon mRNA. Preliminary data show that the major glucagon-containing fragment is processed correctly at the C-terminus while extended N-terminally (Fig. 3) with the GRPP fragment. The apparent correlation of a mixed (fetal) processing pattern of a given glucagonoma and an anorectic phenotype could suggest a combined role of glucagon and Glp1_{7-36amide}. Hepatic infusion of glucagon has been shown to decrease spontaneous meal size in rats [22], whereas Glp1_{7-36amide} requires a central route of administration (intra cerebro-ventricular injection) in order to affect food intake [23]. However, short-term bioassays to measure the anorectic potential of peptide hormones in fasted mice showed no effect of glucagon, Glp1_{7-36amide}, nor their combination in pharmacological doses (80nM/kg), whereas cholecystokinin (CCK-8-S) was a powerful satiety factor at a dose of 40nM/kg [10]. Such an assay may not mimic the tumor effect, however, since the tumor-bearing animals are exposed to grossly elevated circulating hormone concentrations over extended periods. Recently, other glucagon-producing tumors have been derived by a transgenic approach where transformation was preferentially targeted to the intestinal L-cell [24]. Interestingly, these tumors also displayed a mixed processing phenotype but were not associated with severe anorexia [25]. These studies were extended by testing the effect of subcutaneous implantation of three proglucagon-producing cell lines each with different processing patterns [26]. None of them caused anorexia in nude mice although the levels of circulating glucagon-like immunoreactivity reached up to 6000 pM and thus comparable to the levels

found in our glucagonoma-bearing rats during the severely anorectic phase. Since our tumors have retained the anorectic potential when transplanted to immuno-incompetent mice it is likely that substances different from glucagon and Glp1_{7-36amide} are involved in causing the anorectic phenotype. Hitherto unidentified proglucagon derived fragments could still be plausible candidates. Alternatively, a novel polypeptide precursor is produced by the anorectic glucagonomas, which in turn may require a fetal type of processing pathway to liberate the active anorectic substance.

Anorexia, NIDDM, and parabiosis studies between ob/ob and db/db mice

The severity of anorexia caused by the MSL glucagonomas is to our knowledge only matched by an experimental tumor expressing high levels of recombinant TNF [27] and we have presented evidence that the anorectic factor in MSL cells is different from TNF [10]. Interestingly, however, a highly severe anorexia was reported in the classical experiment by Coleman in 1973, where obese (*ob/ob*) mice starved to death following parabiosis with diabetic (*db/db*) mice [12]. When congenic on the C57BL/6J background the *db* and *ob* mice suffered from very similar NIDDM-like syndromes characterized by hyperinsulinemia, hyperglycemia (insulin resistance), hyperphagia and obesity. The distinct genetic defects (autosomal recessive) were ascribed to single loci in *db* and *ob* mice which have been mapped to chromosomes 4 and 6, respectively [28]. During parabiosis the *ob* partner became hypoglycemic, anorectic, and eventually died of apparent starvation, while the *db* partner remained unchanged [12]. A similar fate was observed for normal mice when parabiosed with the *db* mouse [29], while the combination, normal/*ob*, caused the obese partner to eat less and improve its blood sugar and insulin levels to within the near-normal range

[12]. Coleman concluded that the *db/db* mice overproduce a satiety factor but have a defective receptor, while the *ob/ob* mouse is unable to produce the satiety factor but has normal receptor levels. It follows from these studies that NIDDM and severe anorexia may be the two extremes of a common theme, where the important player is an "anorectic factor" possibly encoded by the *Ob*-locus which interacts with its receptor possibly encoded by the *Db*-locus.

Any link between the anorectic factor produced by our glucagonomas and the *db/db* mouse is only speculative, but even earlier studies on normal islet transplantation to *ob/ob* mice carried out by Strautz in 1970 [30] and Gates *et al.* 1972 [31] produced similar phenotypical changes as observed in the "normal/*ob*" parabionts by Coleman, thus suggesting that the anorectic factor was of islet origin [12,30]. It is of further interest that the *db* islet was recently reported to have α -cell hyperplasia, including a frequent subpopulation of cells of fetal appearance expressing more than one hormone [4,32]. In retrospect, such a population might be the source of hyperexpression of a candidate factor involved in satiety regulation.

The fetal nature of our anorectic tumors has been exemplified by the proglucagon processing pattern [10] but also by the extensive polysialylation of the neural cell adhesion molecule, NCAM [33]. All islet cells as well as neurons express NCAM. Polysialylation of NCAM is very pronounced in the fetal brain but absent in adult. When comparing insulinomas and glucagonomas of MSL origin only the glucagonomas have polysialylated NCAM, while the insulinomas express an adult pattern [33]. Such data may support the hypothesis that the glucagonoma-derived factor or the possible *db*-islet-derived factor may be related and that this factor is hyperexpressed by an immature islet α -cell phenotype.

CONCLUDING REMARKS

We have shown that homogeneous insulinomas and glucagonomas [6,10] can be derived from common clonal origin, supporting the existence of a cell lineage relationship between the mature cell types of the islet of Langerhans as proposed by Teitelman and co-workers [2,4]. The transplantable glucagonomas provide a novel tool to study the nature of highly severe anorexia, which is also a characteristic of the glucagonoma syndrome in man. Interestingly, the glucagonoma-bearing rats also suffer from severe adipsia, which may distinguish the syndrome observed in rats from that in man. The incidence of glucagonomas in man is very rare (1 in 20-30 million per year) but a cardinal feature is a characteristic skin rash (necrolytic migratory erythematosis) which occurs in more than 90% of these patients [20,34]. A similar necrosis has not been observed in any of the glucagonoma-bearing rats. The interrelationship between anorexia and adipsia needs further clarification. Identification of the anorectic substance may 1) prove to be a new therapeutic for the treatment of obesity and NIDDM, and 2) allow studies to investigate the possible role of the α -cell (or the L-cell) in the normal hunger response. It is already of major interest that one of the major glucagonoma products, Glp-1_(7-36amide) has proven to be a promising drug in the treatment of NIDDM due to its capacity to reduce circulating glucose levels [35-37].

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