Cloned Cell Lines from a Transplantable Islet Cell Tumor Are Heterogeneous and Express Cholecystokinin In Addition To Islet Hormones

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Abstract. A liver metastasis (MSL) with a remarkable in vitro proliferation potential has been identified in an NEDH rat carrying a transplantable x-ray–induced islet cell tumor. Two insulin-secreting cell lines, MSL-G and MSL-H, with doubling times of 3–5 d were established by repeated limiting dilution cloning. In vivo inoculation of MSL-G cells induced severe hypoglycemia caused by a small but highly heterogeneous tumor as revealed by immunocytochemistry. Whereas most cells stained for the islet hormones, insulin, glucagon, and somatostatin, clustered cells were discovered to contain cholecystokinin (CCK). Additional in vitro–limiting dilution cloning, followed by immunocytochemical characterization, clearly demonstrated the capacity of single cell clones to simultaneously express the same four hormones. Radioimmunoassays with a panel of site-specific antisera of culture supernatants and purified cell extracts showed the MSL-G2 cells to produce, store, and secrete readily detectable amounts of processed and unprocessed CCK. Gastrin was not detected while coexpression of glucagon and CCK were demonstrated. Mutant clones selected for resistance to 6-thioguanine (frequency, 2×10^{-7}) and checked for HAT (hypoxanthine, aminopterin, thymidine) sensitivity retained the capacity for multihormone expression.

We propose that the MSL tumor contains pluripotent endocrine stem cells. The MSL tumor and the MSL-G2 cells in particular will allow studies of not only CCK biosynthesis and processing but also of mechanisms involved in tumor and islet cell differentiation.

Islet cell tumors frequently consist of mixed cell populations producing multiple hormones, although insulin is the major product (2, 16, 22). Whether such tumors are induced by a simultaneous transformation of different mature endocrine cell types or by a single transformation of a pluripotent islet stem cell is not known. The high frequency of occurrence of mixed multihormonal tumors (16, 22) supports the latter hypothesis, assuming that the transformed stem cell has retained the potential to differentiate into various hormone-secreting cells. It also has been speculated that each tumor cell may produce several hormones simultaneously (27, 28). Support for this idea has come from the establishment of clonal endocrine cell lines from an x-ray–induced transplantable islet cell tumor (2), some of which, although initially producing somatostatin, were capable of also expressing insulin (7, 26). In the mature islets of Langerhans in most vertebrates, however, the four major hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) are found in cytologically distinct B, A, D, and PP cells, respectively. Immunocytochemical staining experiments and electron microscopic cell identification have not provided support for the coexistence of any of these hormones within a single cell (16). Two other hormones – thyrotropin-releasing hormone (6) and gastrin – may be detected by biochemical or immunocytochemical techniques in the islets of Langerhans. Gastrin, however, is found only in the fetal and neonatal stage (23). Interestingly, gastrin cells are often detected in insulinomas and other pancreatic endocrine tumors, without clinical signs of hypergastrinemia (16). Additionally, the production of hormones such as growth (hormone)-releasing factor (9, 34, 40), ACTH (16, 22), and calcitonin (38), has been noted in pancreatic endocrine tumors. So far, cholecystokinin (CCK)–producing[1] pancreatic endocrine tumors have not been described.

The identification of a malignant variant of an x-ray–in-

1. *Abbreviations used in this paper*: CCK, cholecystokinin; CTM, complete tissue culture medium.

duced islet cell tumor (2) with an unusually high in vitro proliferation potential as well as a broad hormonal repertoire has allowed us to carry out a long-term study on the nature of multiple hormone-producing tumors. Wild-type and drug-resistant cells from the variant were cloned in vitro and subsequently used for formation of hormone-producing tumors in vivo.

In this report we have characterized these cell lines with respect to their growth properties and potential for gastro-entero-pancreatic hormone expression in vitro and in vivo.

Materials and Methods

Transplantation

Tumors were maintained in vivo by subcutaneous inoculation between the shoulder blades or by intraperitoneal injection in young NEDH rats (80–120 g) of small tumor pieces (1–3 mm^3) from severely hypoglycemic (glucose concentration below 1.5 mM) donors carrying small tumors (0.5–1 g). Similarly, tumors were induced from cloned cell lines by subcutaneous injection of 100 μl serum-free tissue culture medium containing 5–10 × 10^6 cells. After tumor excision, all donors were autopsied for detection of metastases. Blood glucose was measured using Hypocount (Hypoguard Ltd., Woodbridge, UK).

Cell Culture

Primary cultures were prepared by gently mincing freshly excised tumors with forceps and a scalpel in complete tissue culture medium (CTM) composed of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 5–10% fetal calf serum (KC Biological Inc., Lenexa, KS), 5–10% donor horse serum (KC Biological Inc.), and 2 mM L-glutamine and Pen-Strep (100 U/ml) from Gibco. The cell preparation was resuspended followed by sedimentation for 1 min to allow the removal of larger, mostly fibrous, tissue clumps. The supernatant was collected and centrifuged for 5 min at 250 *g*. The pellet was resuspended in CTM and primary cultures were prepared by serial dilution in 96-well (200 μl), 24-well (1 ml), 12-well (2.5 ml), or 6-well (5 ml) plates from Costar (Cambridge, MA). The culture dilutions spanned the cell concentrations of 0 to 500,000 cells/ml to ensure optimal conditions in at least a fraction of the cultures with respect to initial tumor cell concentrations and low fibroblast contamination. Plates were incubated at 37°C in 5% CO_2 in air for 2–4 wk without change of medium. Fibroblast-contaminated cultures could be identified by the color change of medium after 1–2 wk in culture. Residual wells were routinely checked for tumor cell proliferation. Medium was replaced approximately every 3 wk.

Proliferating colonies were isolated in 5 μl using a Pipetman P-20 (Gilson Co., Worthington, OH) equipped with a sterile tip and transferred to 0.5 ml medium in 24-well plates. If colonies attached and spread out during 3–4 wk, they were mechanically disrupted into smaller clumps to allow new colonies to form. A colony density of at least 10 per well was required before conventional trypsination procedures could be used for further propagation. Established cultures were maintained by bi-weekly medium change and trypsinization every 2–4 wk with a split ratio of 1:2 to 1:4.

Cell Cloning

Clonal cell lines were established by the limiting dilution technique. Cell suspensions containing 5–8 cells/ml were distributed into microtiter plates (100 μl/well). Single cell proliferation was supported by 50% (vol/vol) conditioned medium obtained from 1–2-d-old medium from dense primary cultures. Plates were sealed with parafilm strips to allow free CO_2/O_2 exchange and reduce evaporation, and then left in the incubator for up to 1 mo before proliferation was directly visible. Selected clones were expanded as described above.

Trypsinization

Cells were prewashed in Hanks' balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (Gibco) and incubated for 5 min at 37°C with trypsin, EDTA (Gibco). After extensive pipetting, the reaction was stopped by the addition of 1 vol of CTM. Cells were centrifuged and reseeded at the desired density. Several of the MSL cells, especially MSL-G2, attached very firmly

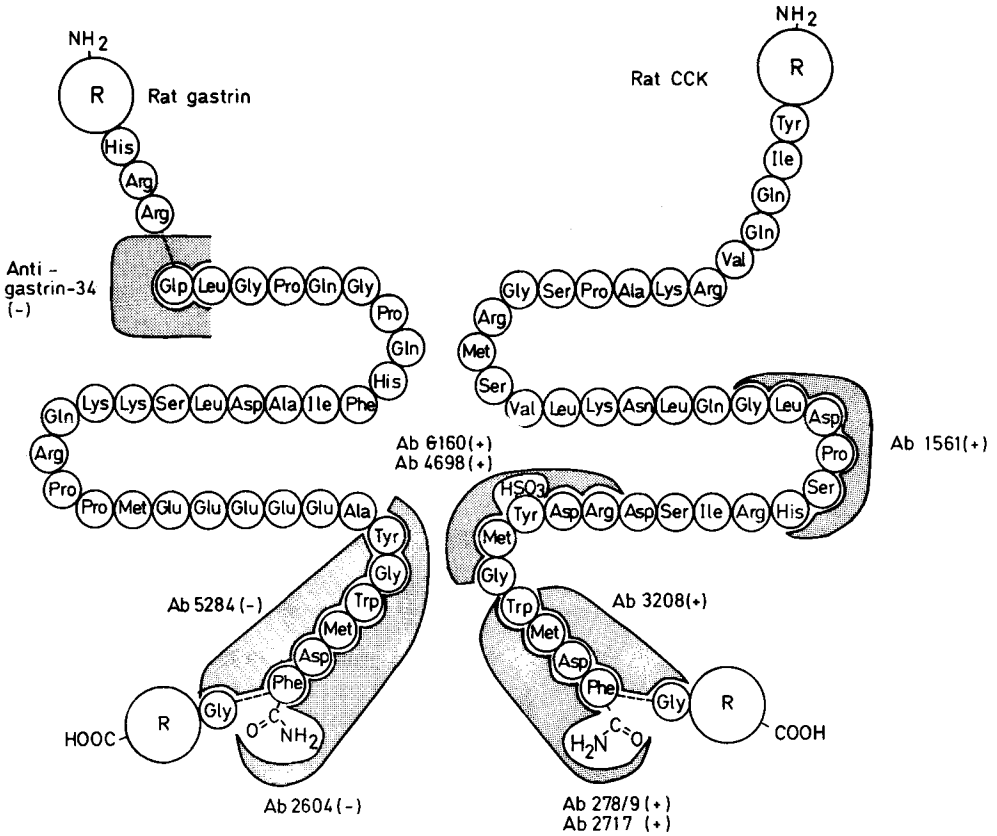


Figure 1. The epitopes recognized by the antisera used for immunocytochemistry and sequence-specific radioimmunoassays are shown for rat gastrin (35) and rat cholecystokinin (3, 4, 15). For references on the antisera, see Materials and Methods. Gastrin and CCK both contain the COOH-terminal antigenic determinant Trp-Met-Asp-Phe-$CONH_2$ recognized by Ab 2717 or Ab 278/9. The immunoreactivity observed towards the MSL cells or secreted products is indicated (+/−). *R* denotes the COOH- or NH_2-terminus of the precursor forms. The rat prepro-CCK, 115 amino acids long, has additionally 12 amino acids COOH-terminal to the amidated Phe-residue (4). The corresponding human preprogastrin (1, 39) is 101 amino acids long with a COOH-terminal extension of 9 amino acids.

to the plastic and required two successive trypsinizations for complete detachment.

Insulin Screening Assay

Insulin assays were performed as described (8, 25) with the following modifications. The use of Sarstedt tubes (no 73.1055) and 96-tube racks (no. 95.1046; Sarstedt, Inc., Princeton, NJ) allowed a semiautomation at the microtiter scale with the use of microtiter plate carriers (Beckman Instruments, Inc., Palo Alto, CA), multichannel pipettes (Flow Laboratories, Inc., McLean, VA), and manifolds (Wheaton Instruments Div., Wheaton Industries, Millville, NJ).

In the assay, 100 μl of monoiodinated insulin (5,000 cpm/tube, obtained from Dr. Bruce H. Frank, Eli Lilly Research Laboratories, Indianapolis, IN) was added to 50 μl tissue culture sample, and 100 μl of guinea pig anti-insulin diluted 1:110,000 (Dako Corp., Santa Barbara, CA; Accurate Chemical & Scientific Corp., Westbury, NY). Bovine γ-globulin (0.22%; Sigma Chemical Co., St. Louis, MO) was present as carrier during the incubation. Carbowax 8000 (12.5%; Fischer Chemical Co., Fair Lawn, NJ) was used to separate bound from free antigen.

Cloning for Drug Resistance

Titration studies were performed using serial dilutions of 6-thioguanine (1 ng/ml to 100 μg/ml; Sigma Chemical Co.) in CTM to optimize selection conditions for particular cell lines. Mass cultures (2–3 × 10^7 cells/75-cm^2 flask) were grown in 25 ml of medium in the presence of the desired concentration of 6-thioguanine with or without ethylmethanesulphonate (150 μg/ml; Sigma Chemical Co.) as mutagen. After ~2 wk, 80% of medium with dead cells was replaced with 10 ml fresh selective medium. Cultures were maintained by a 50% medium change every 4–5 wk until proliferating clones could be observed. Colonies were picked out of bottles using bent pasteur pipettes and propagated according to the method previously mentioned. Surviving clones were tested for HAT (hypoxanthine, aminopterin, thymidine) sensitivity.

Immunocytochemistry

Cell cultures growing on coverslips (9 × 9-mm, Bellco Glass, Inc., Vineland, NJ) were fixed in 1% paraformaldehyde in 0.05 M sodium phosphate buffer (pH 7.4) (18) and could be stored for prolonged times in fixative without loss of immunoreactivity. The cells were permeabilized as described (18). In addition, solid tumors were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for at least 24 h, soaked in 20% (wt/vol) sucrose in the same buffer overnight, and then quenched in melting Freon-22 as described (18). Cryostat sections were cut at 8 μm at −20°C. Permeabilized coverslips and cryostat sections were stained by previously detailed procedures (18), using the following: (*a*) rabbit gastrin/CCK antiserum No. 2717, recognizing the COOH-terminal tetrapeptide amide common to gastrin (Fig. 1) and CCK (20); (*b*) rabbit CCK-antiserum 1561, recognizing the NH_2-terminal 15–20 region of CCK-33 and devoid of cross-reactivity to gastrin (Fig. 1) (32, 33); (*c*) rabbit CCK-antiserum 4698, recognizing the NH_2-terminal tetrapeptide of CCK-8 and devoid of cross-reactivity to gastrin (Fig. 1) as detailed elsewhere (20, 29); (*d*) rabbit gastrin-34-specific antiserum, specifically recognizing the NH_2-terminal sequence of gastrin-34 (Fig. 1), kindly donated by Dr. N. Yanaihara, Shizuoka, Japan, and described in detail elsewhere (13); (*e*) rabbit anti-glucagon sera Nos. 4304 and 4316, kindly donated by Dr. Jens Holst, Dept. of Physiology, Panum Institute, Copenhagen. Antiserum 4304 reacts with both pancreatic glucagon and with larger glucagon precursor forms in-

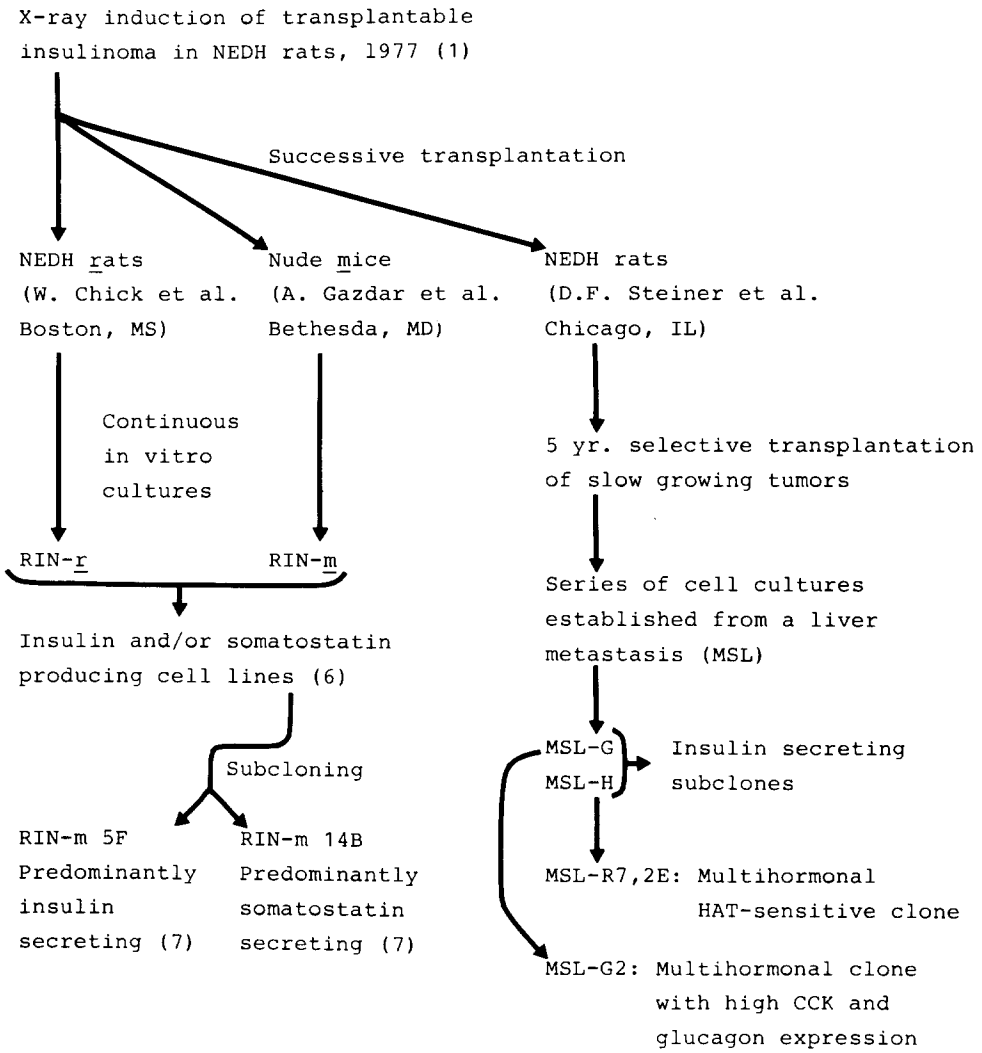


Figure 2. Origin of the MSL cells. The relationship to RIN cell lines (2, 7, 26) is illustrated.

cluding glicentin, whereas serum 4316 reacts exclusively with pancreatic-type glucagon (19); (*f*) rabbit anti-somatostatin antiserum R213/3, previously described in detail (21); (*g*) rabbit anti-human pancreatic polypeptide serum No. 615-1054B-216-2, kindly donated by Dr. R. E. Chance (Eli Lilly Research Laboratories, Indianapolis, IN) and previously characterized (24); (*h*) guinea pig anti-insulin serum, kindly donated by Dr. J. Holst; (*i*) guinea pig anti-CCK antiserum No. 4488, raised against CCK-33 and reacting as described (29); (*j*) guinea pig anti-motilin serum, a kind gift of Professor N. Yanaihara; or (*k*) rabbit anti-human growth hormone serum kindly donated by Dr. K. Hanssen, Department of Medicine, Akers Sykehus, Norway, and previously characterized (17). All antisera were applied at their optimal dilutions for 24 h at 4°C as described (18). The site of antigen-antibody reaction was revealed either by the peroxidase-antiperoxidase procedure of Sternberger (37) or by indirect immunofluorescence using fluorescein isothiocyanate-(FITC-) or tetramethylrhodamine isothiocyanate-(TRITC-) labeled antibodies (18). Immunofluorescence preparations were examined in a Leitz Orthoplan epifluorescence microscope with selective interference filters and mercury/xenon burners for excitation of FITC and TRITC, at 490 nm or 546 nm, respectively.

Controls included conventional staining controls as recommended (37) as well as specificity controls using antisera preabsorbed against synthetic human gastrin I (ICI), synthetic somatostatin-14 (Peninsula Laboratories, Inc., Belmont, CA) purified porcine glucagon (NOVO, Bagsvaerd, Denmark), synthetic tetragastrin and gastric inhibitory polypeptide (Peninsula Laboratories, Inc.), 99% pure porcine CCK-33 (a kind gift from Professor V. Mutt, Department of Chemistry, Karolinska Institute, Stockholm, Sweden), and insulin (Nordisk Gentofte A/S, Gentofte, Denmark). All stainings were specific according to these criteria and could be abolished only by preabsorption against the appropriate but not unrelated peptides.

Indirect immunofluorescence was used in double-staining experiments to test for the joint presence of two hormones in the same cell. Permeabilized cells on coverslips were stained with rabbit anti-glucagon serum No. 4304, whereafter the site of antigen-antibody reaction was revealed with TRITC-labeled anti-rabbit IgG that had been preabsorbed against guinea pig IgG coupled to cyanogen bromide-activated Sepharose 4B beads. After rinsing, guinea pig anti-CCK serum No. 4488 was applied and finally, FITC-labeled anti-guinea pig IgG, preabsorbed against rabbit IgG-Sepharose beads, was applied. Specimens were analyzed in the above fluorescence microscope and pictures at selective FITC or TRITC excitation were taken on Kodak Ektachrome Professional Daylight film of 200 ASA. Controls included those applied above for single staining. In addition, combinations of primary rabbit antiserum and FITC-labeled anti-guinea pig IgG and of primary guinea pig antiserum and TRITC-labeled anti-rabbit IgG produced negative results documenting the species specificity of the second fluorochromed antibodies. Finally, reversal of the staining sequence (guinea pig sequence first) produced identical results.

Differential counts (n >300) were performed to quantitate positively stained cells in the peroxidase-antiperoxidase method. This was carried out at different tissue culture passages using the following antibodies: anti-COOH-terminal gastrin/CCK Ab 2717; anti-glucagon Ab 4304; anti-somatostatin Ab R213/3, and anti-insulin.

Sequence-specific Radioimmunoassays

Development and characteristics of the four radioimmunoassays specific for different sequences of rat preproCCK (using antisera nos. 1561, G-160, 278, and 3208) and of the two control radioimmunoassays specific for different sequences of preprogastrin (using antisera nos. 2604 and 5284) have been described in detail elsewhere (29, 32). Briefly, Ab 1561 binds sequence 85–90; Ab G-160 binds sequence 95–99, Ab 278 binds sequence 99–103; and Ab 3208 binds sequence 100–104 of rat preproCCK (Fig. 1). Gastrin antiserum Ab 2604 binds the COOH-terminal octapeptide amide of rat

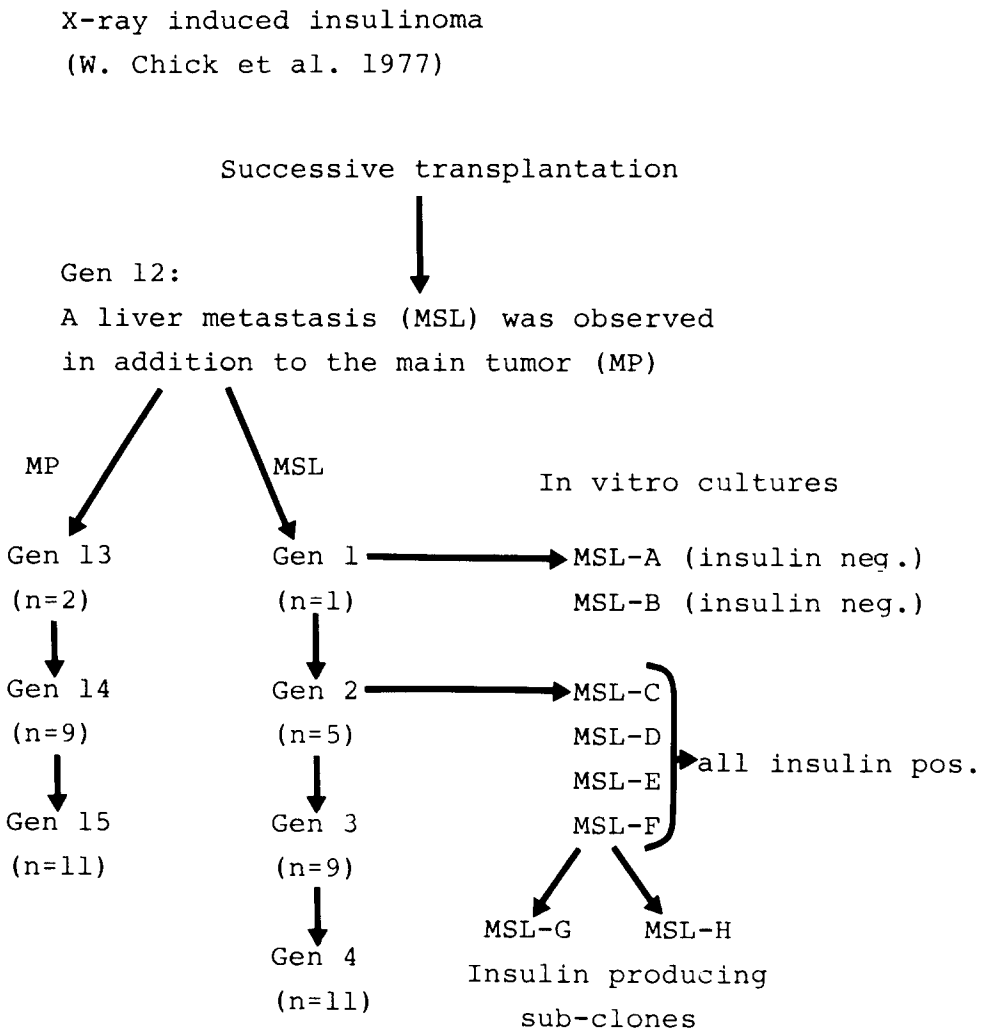


Figure 3. Subsequent transplantations and tissue cultures derived from the animal with the observed metastasis. The corresponding generations are indicated for MP and MSL (n is number of animals per generation).

gastrin-17 and Ab 5284 the corresponding glycine-extended COOH-terminus (Fig. 1). Neither of the two latter gastrin antisera cross-react with any products of preproCCK. The radioimmunoassays were performed as described in detail elsewhere (29, 32).

Results

During routine propagation of the x-ray–induced transplantable islet cell tumor (2) in NEDH rats, involving selective transplantation of small tumors from severely hypoglycemic animals, we observed a tumor nodule in the liver which was assumed to represent a metastasis (Fig. 2). In this single rat, which had received the tumor tissue sample intraperitoneally, the main tumor mass was attached to the pancreas. The metastasis was clearly embedded within the liver tissue.

Separate propagation in vivo of the metastasis (MSL) as well as of the main tumor (MP) from the same animal by subcutaneous transplantation to the back resulted in a series of insulin-producing tumors (Fig. 3). However, in successive generations of the MSL tumor, the insulin expression was unstable as indicated by gradually increasing tumor size, where large cystic tumors developed in the fourth generation with only a single animal becoming hypoglycemic (Table I). In contrast, transplants from the main tumor consistently produced small-sized tumors associated with severe hypoglycemia in the subsequent generations. Interestingly, no metastases were observed in either MSL or MP recipients at the time of tumor removal (n=26 and 22, respectively). The MSL tumor displayed a remarkably high in vitro proliferation potential as compared to the original x-ray tumor and the MP-variant (Table I). The relationship of MSL cultures and the RIN cell lines (7) of similar origin is shown in Fig. 2.

Primary Cultures

All tumor cell preparations from the MSL tumor yielded proliferating primary cultures (n = 5), whereas none of the attempts using identical procedures with the MP tumor were successful (n = 20) (Table I). It was observed that fibroblast contamination strongly reduced attachment of the primary tumor cells, thus inhibiting subsequent proliferation. Our serial dilution culture procedure ensured, however, that a certain fraction of primary microcultures from a particular tumor would be devoid of fibroblasts.

Establishment of Insulin-producing Cell Cultures

The simple screening assay for immunoreactive insulin allowed large-scale screening to identify insulin-secreting microcultures. A second generation MSL tumor causing severe hypoglycemia was used to start more than 200 secondary cultures, each containing a proliferating colony, which was isolated from insulin-positive primary cultures. Four secondary cultures with insulin concentrations greater than 20 ng/ml after 1 wk in 1-ml cultures were selected for expansion and cryopreservation (MSL-C, -D, -E, and -F; Fig. 3). One of these cultures (MSL-F) was subjected to limiting dilution cloning twice, to establish two insulin-secreting cell lines; MSL-G and MSL-H. The doubling times of the various cultures varied between 3 and 6 d.

Multihormone Expression In Vivo by the Clonal Culture MSL-G

Subcutaneous inoculation of MSL-G cells in NEDH rats induced small, solid tumors (200–500 mg) associated with severe hypoglycemia. Immunocytochemical characterization of MSL-G tumor sections showed that, in addition to numerous insulin cells, discrete small areas in the tumor also contained glucagon, somatostatin, and CCK immunoreactive cells (Fig. 4, somatostatin staining not shown).

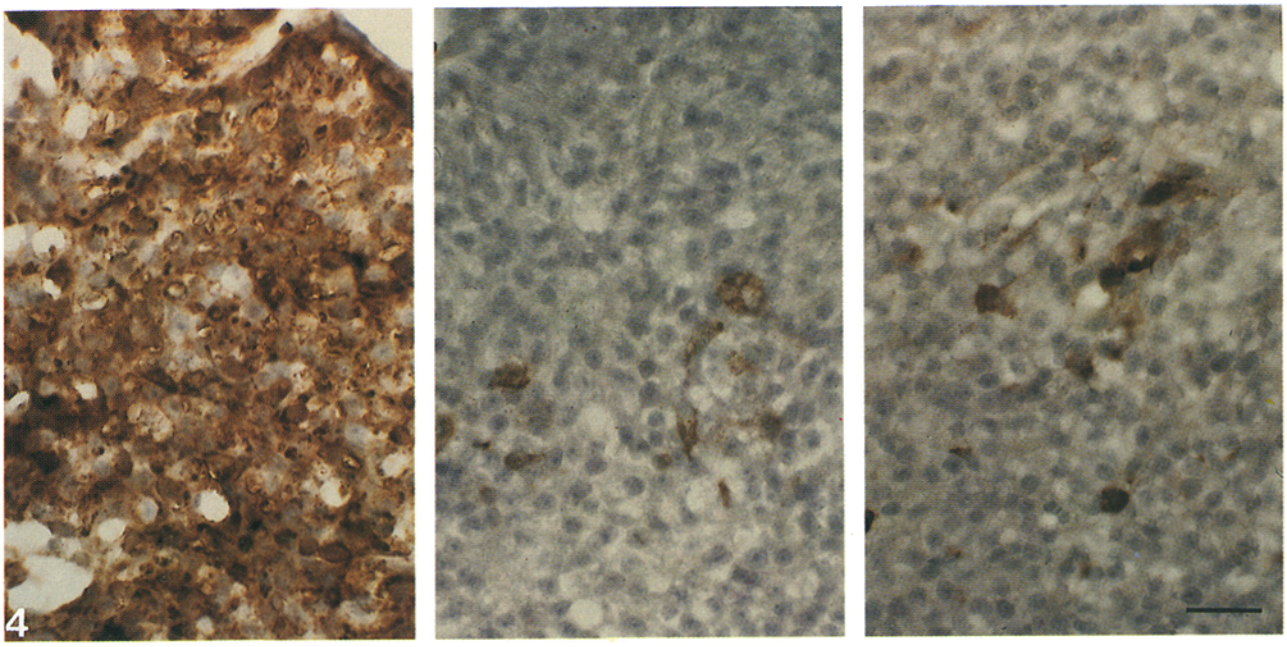
The Cholecystokinin/Glucagon–producing MSL-G2 Variant

After ~8 mo of continuous culture of the MSL-G cells, a morphologically distinct and slightly faster growing variant with a doubling time of ~4 d was observed (Fig. 6). Isolation and propagation of this variant (MSL-G2) resulted in a continuously constant expression of this particular phenotype for >9 mo. The MSL-G2 culture gradually ceased to secrete detectable amounts of insulin into the medium. Immunocytochemical analysis of monolayer cultures of MSL-G2 (Fig. 7, *A–C*) as well as of its subclone, Cl-3 (Fig. 7, *D–F*) revealed numerous glucagon and COOH-terminal gastrin/CCK-like immunoreactive cells (Fig. 1). The use of non–cross-reacting antibodies towards gastrin and CCK (Fig. 1) demonstrated that the immunoreactivity was due to CCK, whereas gastrin-specific staining could not be detected. These results were confirmed by radioimmunoassay on supernatants (Table II) and purified extracts (Fig. 8) of MSL-G2 cells. Larger forms of presumably non-amidated CCK accumulate in the medium under normal culture conditions (Table II) whereas smaller amidated forms are found in cell extracts thus assumed to be the major stored products (Fig. 8). This was also reflected in supernatants of cultures stimulated to secrete stored immunoreactivity (Table II). The reaction of Ab 1561 (Fig. 1) to the Cl-3 cells is shown in Fig. 7 *F*. The fractions of MSL-G2 cells positive for glucagon or CCK were of the same size and varied from 50 to 80% throughout a 6-mo cul-

Table I. Comparison of MP and MSL Tumor Cells

	Tumor generation						
	1 MSL	MP	2 MSL	MP	3 MSL	MP	4 MSL
Animals per generation	1	2	5	9	9	11	11
Hypoglycemic animals (%)	1	2	5	9	5	11	1
Continuous in vitro growth/culture attempt	1/1	0/2	2/2	0/9	2/2	0/11	–
Tumor size (g)	<1	<1	4–6	<1	>10	<1	>10

The size, the ability to induce hypoglycemia in vivo, and the proliferation potential in vitro is shown for MSL and MP tumors in subsequent generations. Large tumors were cystic.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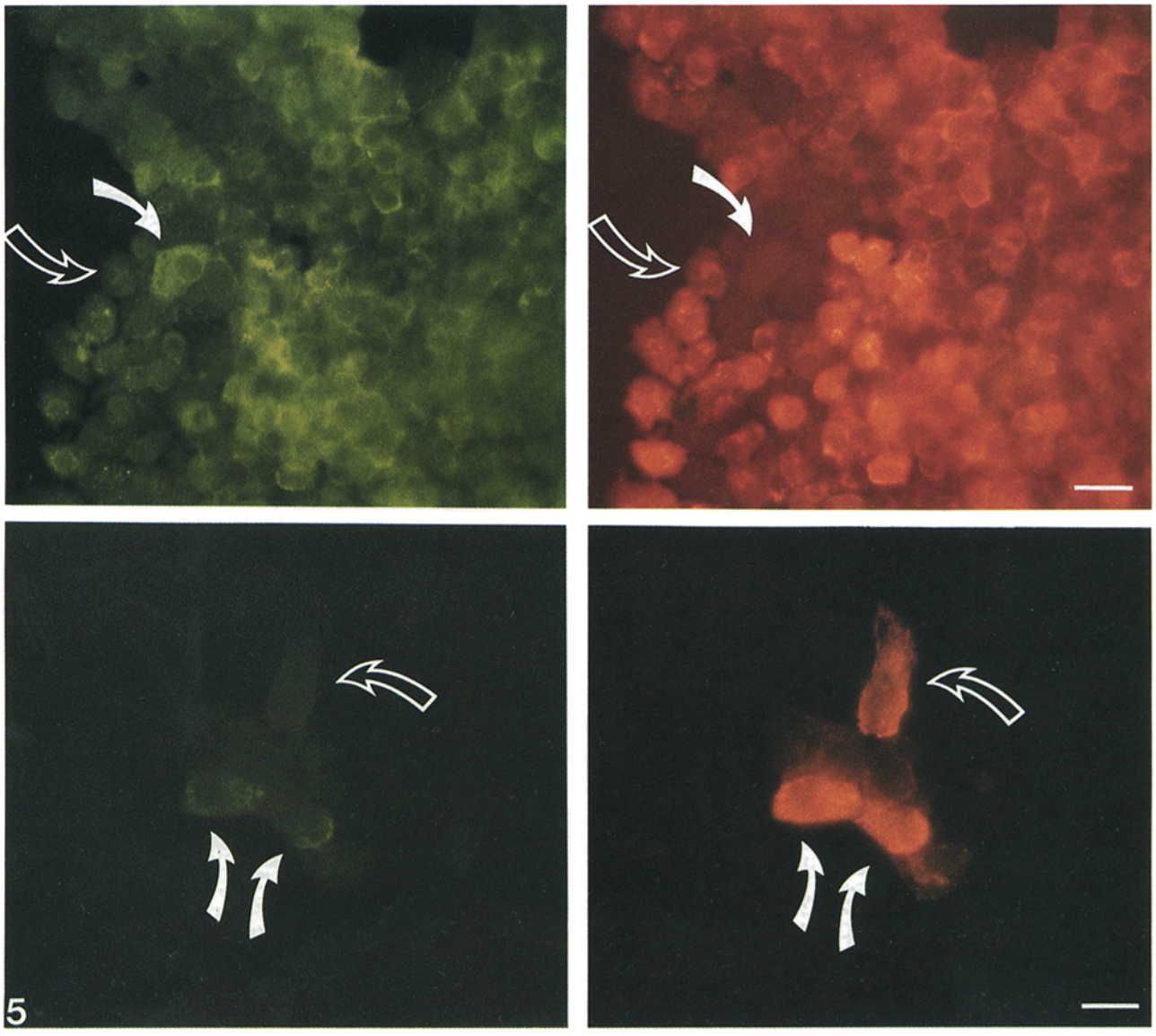
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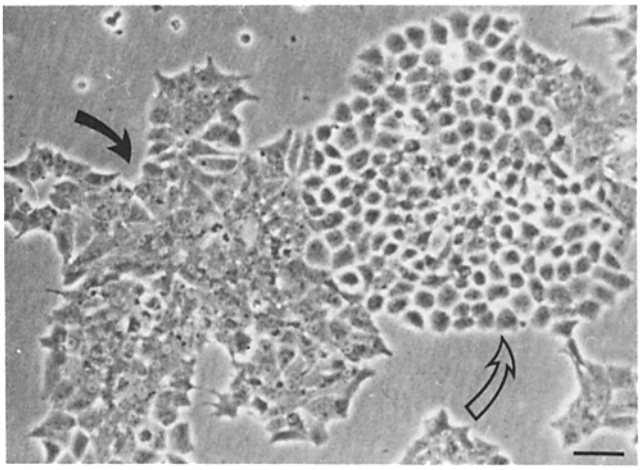

Figure 6. Phase-contrast microscopy of the original MSL-G cells (*filled arrow*) with the MSL-G2 variant (*open arrow*). Bar, 30 μm.

ture period (Table III). Somatostatin- and insulin-positive cells were present throughout the culture period but in much lower numbers (Table III). The distribution of the expression of the two hormones was rather remarkable since groups of typically 2–4 strongly stained cells were scattered in areas of negative cells (as shown in Fig. 7 *C*). A similar profile of hormone expression was found in Cl-3. Double-staining immunofluorescence analyses with glucagon and CCK antisera raised in a rabbit and a guinea pig, respectively, showed that the majority of the positive cells contained both types of immunoreactivity. However, a minority of cells in the same culture stained for only either glucagon or CCK (Fig. 5). MSL-G2 and Cl-3 were negative for the other hormones tested including motilin, growth hormone, and pancreatic polypeptide.

Other Multihormone-producing Cell Lines

The immunocytochemical analysis of the in vitro insulin-producing clone MSL-H revealed a mixed hormone expression including the same four hormones as above (Table III). Since the limiting dilution technique does not provide the ultimate guarantee of monoclonal origin, we performed drug selection experiments to confirm our data. Selection by 6-thioguanine was used to obtain HAT (hypoxanthine, aminopterin, thymidine)-sensitive cultures and allowed us to isolate a series of insulin- and non-insulin–secreting clones. The frequency of proliferating 6-thioguanine resistant clones in the MSL-H culture was extremely low (2.5×10^{-7}) and independent of the presence of ethylmethanesulphonate. One insulin-secreting clone (MSL-R7,2E) tested so far retained the capacity for multihormone expression, although the fraction of positive cells for a particular hormone was very low (Table III). However, the intensity of staining of the few positive cells with glucagon, CCK, and somatostatin, respectively, was strong (Fig. 7, *G*, *H*, and *J*). Among the non-insulin–secreting cell lines tested so far, including MSL-A, (Table III) all expressed glucagon and CCK.

Table II. Quantitation of CCK Peptides by Radioimmunossay

Standard	Tracer	Anti-serum	Accumulation in 2 d		Release in 90 min	
			pmol/10⁶ cells		*pmol/10⁶ cells*	
			I	II	I	II
CCK 33	^{125}I-CCK 33	1561	17.4	14.0	2.9	3.8
CCK-8-gly	^{125}I-G13-gly	3208	5.6	6.7	3.2	2.5
CCK-8 (S)	^{125}I-CCK-33	278/9	3.0	2.8	15.7	>10*
CCK 8 (S)	^{125}I-CCK-33	G160	3.0	5.0	1.2	1.9
G13-gly	^{125}I-G13-gly	5284	0	0	0	0
G17	^{125}I-G17	2604	0	0	0	0

Supernatants of cultured cells were assayed for CCK/gastrin-like immunoreactivity. Culture conditions were as follows: 5×10^5 cells in 4 ml (I) and 10^6 cells in 4.7 ml were incubated for 2 d in normal medium. Medium was collected for assay and cells were incubated 90 min with serum-free medium. This medium was known to reduce cytoplasmic hormone content based on immunocytochemical results, and thus assumed to stimulate secretion of stored products.

* Lowest dilution tested gave full displacement in assay. Antiserum 3208 recognizes COOH-terminal glycine-extended gastrin or CCK which are the immediate precursors of amidated gastrin and CCK (Fig. 1). G13-gly is COOH-terminal glycine-extended gastrin-13. Specificities of antisera are shown in Fig. 1. The antisera 5284 and 2604 are gastrin specific.

Table III. Immunocytochemical Results

Clone	Passage number	% cells reactive with antiserum aganist				
		Insulin	Glucagon	Somatost.	PP	CCK
MSL-G	10	2	33	10	0	30
MSL-G2	11	2	78	5	nt	78
MSL-G2	13	0.2	72	nt	nt	67
MSL-G2	14	0.2	58	4	nt	59
MSL-G2	19	nt	48	nt	nt	52
MSL-CL3	17	1	46	6	nt	30
MSL-A	6	0	9	10	0	1
MSL-H	17	1	1	6	nt	<0.1
MSL-R7, 2E	6	<0.1	4	3	0	4

Results of differential counting of positively stained cells using antisera against the hormones indicated.

Discussion

We have identified a liver metastasis (MSL) of an x-ray–induced pancreatic islet cell tumor (2). The metastatic cells expressed an enhanced potential for in vitro proliferation. This is in accordance with the observation that malignant cells can be cultured more readily in vitro as compared to their normal and benign counterparts (11, 36). To our knowledge, metastatic spreads have not previously been reported for this tumor. Interestingly, no metastases were identified in any of the rats carrying the MSL tumor ($n = 26$). However, two MSL cell lines (MSL-A and MSL-G) have occasionally produced

Figures 4 and 5. (*Fig. 4*) MSL-G tumor sections stained for insulin (*left*), CCK (*center*), and glucagon (*right*). Bar, 20 μm. (*Fig. 5*) Double-staining experiments. (*Left panels*) CCK. (*Right panels*) Glucagon. In top panels, most cells stain for both hormones simultaneously. However, few cells predominantly contain either glucagon or CCK (*open arrows* and *filled arrows*, respectively). In bottom panels, two cells in a small colony contain both hormones (*filled arrows*) whereas one cell only stains for glucagon (*open arrow*). Bar, (*top panels*) 20 μm; (*bottom panels*) 10 μm.

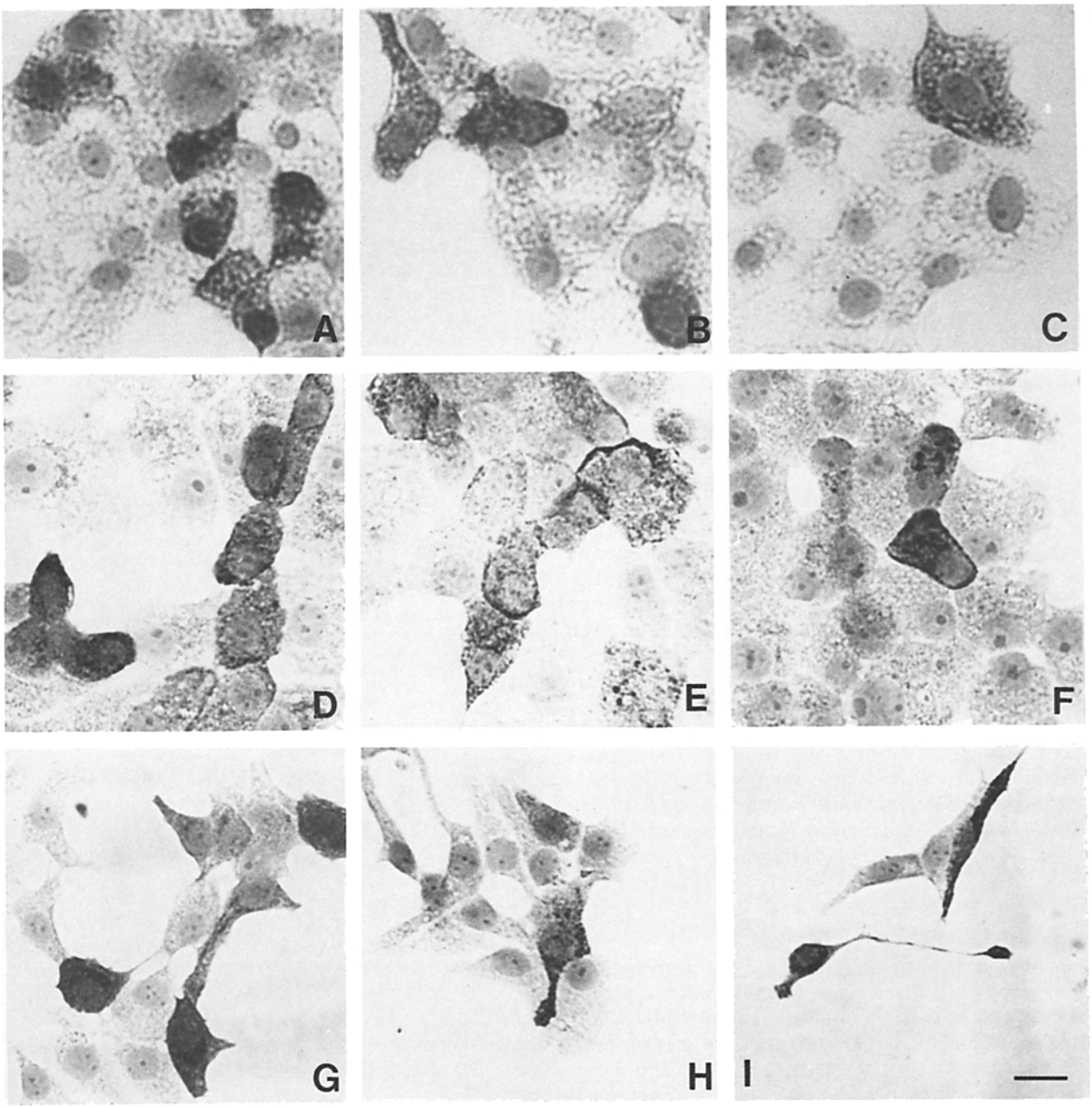

Figure 7. Immunocytochemical staining of monolayer cell cultures. MSL-G2 (*A–C*), MSL-C13 (*D–F*), and MSL-R7,2E (*G–I*) were stained for glucagon (*A*, *D*, and *G*), somatostatin (*I*), insulin (*C*), COOH-terminal gastrin/CCK (Ab 2717) (*B*, *E*, and *H*), and CCK (Ab 1561) (*F*). Bar, 10 μm.

metastases when grown subcutaneously in vivo. In two rats carrying MSL-A tumors, multiple small cystic tumors were observed in the liver. One rat carrying an MSL-G tumor developed two small solid insulin-producing metastases, one in each lung (Madsen, O. D., unpublished observations).

All the clonal MSL-cell lines studied show a multihormonal expression. Interestingly, RIN cell clones of same origin (Fig. 2) were also found to express insulin and somatostatin (7, 26). These results strongly suggest that the original tumor arose from a transformed pluripotent islet stem cell, or that transformation lead to the dedifferentiation of a mature cell to a pluripotent stage. The fact that most human pancreatic islet cell tumors are multihormonal (16, 22) is consistent with either of the above hypotheses regarding the mechanism of islet tumor genesis. The MSL cells thus allow studies of in vitro differentiation processes associated to the expression of particular hormones.

The demonstration of the presence of CCK and not gastrin reveals a hitherto undescribed hormone expression by transformed pancreatic islet cells. The MSL-G2 cells in particular have been shown to produce and secrete various forms of processed and unprocessed CCK. The hormone regulates gall bladder contraction (12), secretion of pancreatic enzymes (10, 14), and has neurotransmitter activity (5; see 31 for review). As with other regulatory peptides, preproCCK undergoes a series of posttranslational modifications leading to a heterogeneous group of CCK peptides with different biological activities (30, 31). The posttranslational modi-

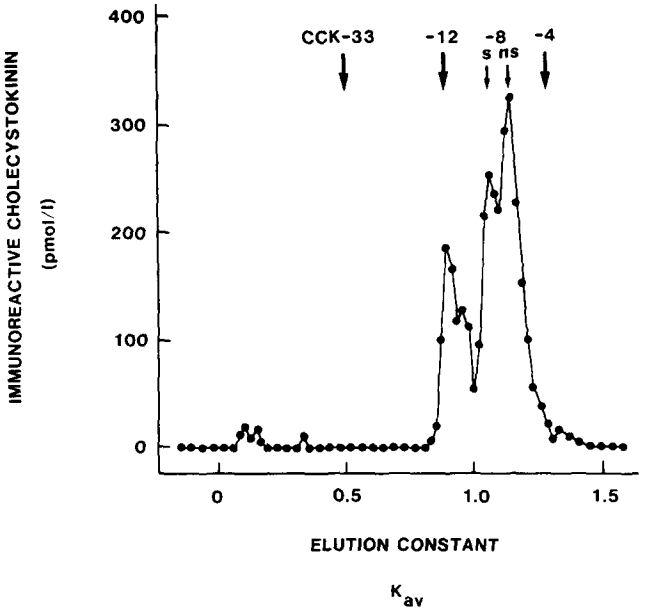

Figure 8. Gel chromatography of a boiling water extract of MSL-G2 cells. 1-ml extract (10^6 cells) was applied to a calibrated Sephadex G 50-superfine column (10 × 1,000 mm) eluted at 4°C in 0.02 M sodium veronal (pH 8.4) containing 0.1% bovine serum albumin at a flow rate of 5 ml/h. 1-ml fractions were collected and measured by cholecystokinin radioimmunoassays as described (29, 32). The measurements shown here (●–●) were performed with the COOH-terminal-directed CCK-antiserum 278/9, which binds CCK-4, -8, -12, and -33 with equimolar potency.

fications of CCK include proteolytic processing, tyrosin O-sulphatation, and α-carboxy-amidation. The MSL cells will allow studies of mechanisms involved in CCK biosynthesis and processing in vitro. The demonstration of high concentrations of processed CCK forms underlines the considerable differentiation potential of the MSL cells.

The establishment of HAT-sensitive pluripotent pancreatic endocrine cells such as MSL-R7,2E provides a vehicle by which novel hormones may be expressed using cell fusion techniques.

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