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Signet Ring Stomach Cancer: Morphological Characterization and Antigenic Profile of a Newly Established Cell Line (Mz-Sto-1)

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Abstract—*A human gastric signet ring cancer cell line (Mz-Sto-1) was established in tissue culture from the ascites fluid of a 54-year-old patient. The tumor cells growing in tissue culture exhibit the morphological characteristics of signet ring cells in phase contrast and transmission electron microscopy. Mz-Sto-1 cells grow as monolayer with a population doubling time of 28–36 hr during exponential growth phase and show a chromosome number between 72 and 74. In the cellular DNA of Mz-Sto-1 cells no amplification of 19 oncogenes studied is observed, c-myc included. Mz-Sto-1 cells secrete 150–250 ng CEA per 10^7 cells in 3 days, but no AFP. In addition Mz-Sto-1 cells and 2 already established gastric cancer cell lines MKN-28 and MKN-45 express HLA- and blood group related antigens (A, Lewis). HLA-DR antigens, which are regularly detected on normal stomach epithelium, are not found on any of the 3 cultured gastric cell lines. Mz-Sto-1 cells represent the first human gastric cancer cell characterized ultrastructurally as signet ring cells. This line will be a valuable tool to study the biology and genetics of gastric carcinoma, to test cytostatic drugs and to define new antigenic markers for stomach cancer.*

INTRODUCTION

STOMACH cancer has not only a high incidence in Japan but is also prevalent in Europe. The biology and genetics of these tumors has not been studied very extensively. One reason is obviously the lack of appropriate *in vitro* culture systems. Only a few gastric cancer cell lines have been established, most of them in Japan [1–12]. Some of those cell lines are no longer being maintained [4, 6, 7]. Only very few are still available for ongoing research. So far, signet ring cancer cell lines which have been well-defined ultrastructurally are missing. In this report we describe morphological, chromosomal and antigenic features of a newly established signet ring carcinoma cell line (Mz-Sto-1), derived from the ascites fluid of a 54-year-old Caucasian male with gastric cancer. In addition, amplification of *c-myc* oncogene DNA is studied in 3 gastric cancer cell lines (Ms-Sto-1, MKN-28, MKN-45), because 2 recent reports [13, 14] described its amplification in gastric carcinoma. The cell surface phenotype of Mz-Sto-1, MKN-28 and MKN-45 gastric cancer cell lines is defined employing a series of monoclonal antibodies.

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PATIENT, MATERIALS AND METHODS

Patient

Patient G, 53-years-old, suffered from cancer of the stomach, 20 years after Billroth I surgery, because of duodenal ulcers. Gastrectomy was performed. Histological examination of the primary tumor revealed a moderately well-differentiated adenocarcinoma with islets of signet ring cells, which stained PAS-positive (periodic acid Schiff reagent) by routine histochemistry. Chemotherapy was initiated because the tumor was not resected completely. However, the tumor progressed and ascites developed about 1 year after initial diagnosis. Ascites fluid was obtained for tissue culture with the patient's consent and established as a permanent cell line.

Tissue culture

Ascites cells were spun down at 150 ***g*** for 10 min, resuspended in a 1 : 1 mixture of ascites fluid and CMRL tissue culture medium (GIBCO, NY, U.S.A.), supplemented with 15% fetal bovine serum (FBS, heat inactivated at 56° C for 30 min), 2 mM glutamine, 0.01 U/ml insulin, 1% non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin transferred into 75 cm^3 tissue culture flasks (Nunc, Roskilde, Denmark) and kept at 37°C and 5% CO_2 atmosphere. The Mz-Sto-1 monolayer cells were detached by 0.05% EDTA (ethylenediamine tetraacetic acid) in Ca^{2+} and Mg^{2+} free PBS and subcultured. Fibroblasts and mesenchymal cells were selectively removed by repeated short EDTA treatment. Growth kinetics were determined by seeding a fixed cell number into parallel tissue culture flasks on day 0. In 24 hr intervals the cells of 1 flask were detached and counted by hemocytometer. Calculation of the cell doubling time was performed from the logarithmic portion of the growth curve. MKN-28 and MKN-45 were described in Reference [2] and were kindly provided by Dr. Schmiegel, Medical Clinic, Eppendorf, 2000 Hamburg, W. Germany. All cells were regularly tested for fungal, bacterial and mycoplasma infections and infected cultures discarded. The 33258 Hoechst stain was employed for mycoplasma testing [15].

Microscopy

Phase contrast micrographs of the cultured stomach carcinoma cell line were taken by an inverted microscope (Zeiss, Oberkochen, W. Germany). Transmission electron micrographs were prepared as reported previously [16]. The cells for scanning electron microscopy were prepared according to standard methods. Briefly, the cells were rinsed in Sorensen buffer 3 times for 30 min after fixation in 2.5% glutaraldehyde for 30 min and then subjected to freeze-drying. After carbon-coating the cells were viewed with the PSEM (Philips Scanning Electron Microscope).

Chromosomal analysis

Mitotic peaks were calculated, based on growth characteristics and cell cycle duration, and enhanced first by a feeding rhythm, eventually by application of 0.5 μg colcemide per ml 4 hr prior to harvesting. After a hypotonic shock of 10 min with 0.05 M potassium chloride, cells were fixed with ethanol–acetic acid 3 : 1, washed several times, and spread by an air-drying procedure for chromosome analysis.

Heterotransplantation

Athymic mice (NMRI nu/nu) at 4 weeks of age were inoculated subcutaneously with 2×10^7 Mz-Sto-1 cells into the back and observed for tumor growth up to 2 months thereafter.

Extraction of cellular DNA, oncogene probes and hybridization procedures

Total high molecular weight DNA of the various cell lines was prepared by lysis in 3% *N*-lauroxylsarcosine sodium salt, 0.07 M Tris pH 8.0, 0.025 EDTA and proteinase K treatment followed by phenol and chloroform–isoamylalcohol (24 : 1) extraction using standard protocols [17].

Ten μg of cellular DNA were digested by a 4- to 5-fold excess of the restriction enzymes under the conditions specified by the commercial suppliers. Restriction-endonuclease fragments were subjected to electrophoresis in 0.7%–1% agarose gels using 40 mM Tris–acetate, 2 mM EDTA pH 7.8 as running buffer. DNA was transferred from the gels onto nitrocellulose filters according to the method of Southern [18]. For prehybridization nitrocellulose membranes were incubated at 42° C in 5 × SSC (1 × SSC consisting of 0.15 M NaCl, 0.015 M sodium citrate), 20 mM sodium phosphate buffer pH 6.5, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.5 mg/ml denatured t-RNA and 50% formamide for hybridization under stringent conditions. Hydridization was performed at 42° C. The hybridization solution was the same as that used for prehybridization except that it contained only 0.1 mg/ml t-RNA plus the ^{32}P-labelled DNA. After hybridization filters were washed 3 × 30 min in 2 × SSC, 0.1% SDS at 68° C. Filters were autoradiographed at −70° C on Kodak XOMAT-AR 5 films using intensifying screens.

Preparation of cloned DNA probes was performed by cleaving the inserts off the vector, separation by electrophoresis and subsequent electroelution [17] and NACS Prepac mini-column concentration as recommended by the manufacturer (BRL, Gaithersburg, U.S.A.). Purified DNAs were radioactively labelled with ^{32}P by nick-translation [19]. The reaction was carried out with 500 ng plasmid DNA or 1 μg cellular DNA. Specific activities were about 10^8 cpm/μg. Approximately 10^5 cpm/cm^2 filter area were used in most hybridization reactions.

Plasmid DNA clones of *c-erbA*$_1$, *c-erbA*$_2$, *c-erb-B*, *v-raf*, *v-myb*, *v-sis*, *c-N-ras*, *c-Ha-ras*, *c-Ki-ras*, *v-rel*, *c-myc*$_1$, *c-myc*$_2$, *c-myc*$_3$, *v-src*, *v-fgr*, *v-yes*, *v-yes*, *v-fms*, *v-fos*, *v-mos*, *v-abl* were kindly provided by Drs. H. zur Hausen, L. Gissman at the Deutsches Krebsforschungszentrum and R. Müller EMBL, Heidelberg. Two hundred and fifty ng of plasmid DNA of each oncogene probe were cleaved, insert and vector DNA separated by electrophoresis and blotted for preliminary screening of gene amplification. This procedure permits the detection of 10 genome equivalents per cell [20].

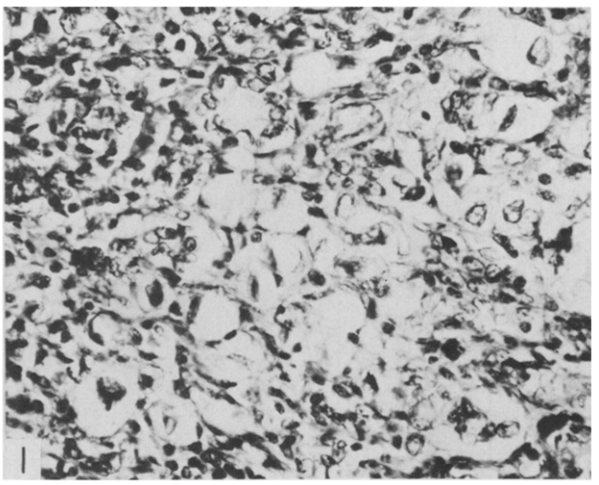


Fig. 1. *Hematoxylin–eosin staining of the primary tumor specimen: moderately–well-differentiated adenocarcinoma with islets of signet ring cells. Magn.* × 336.

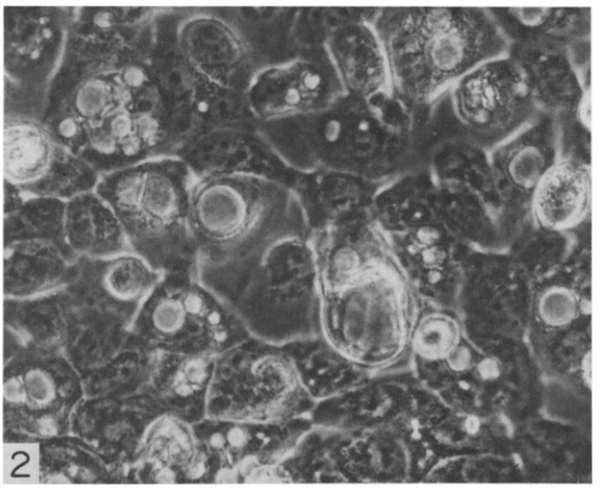
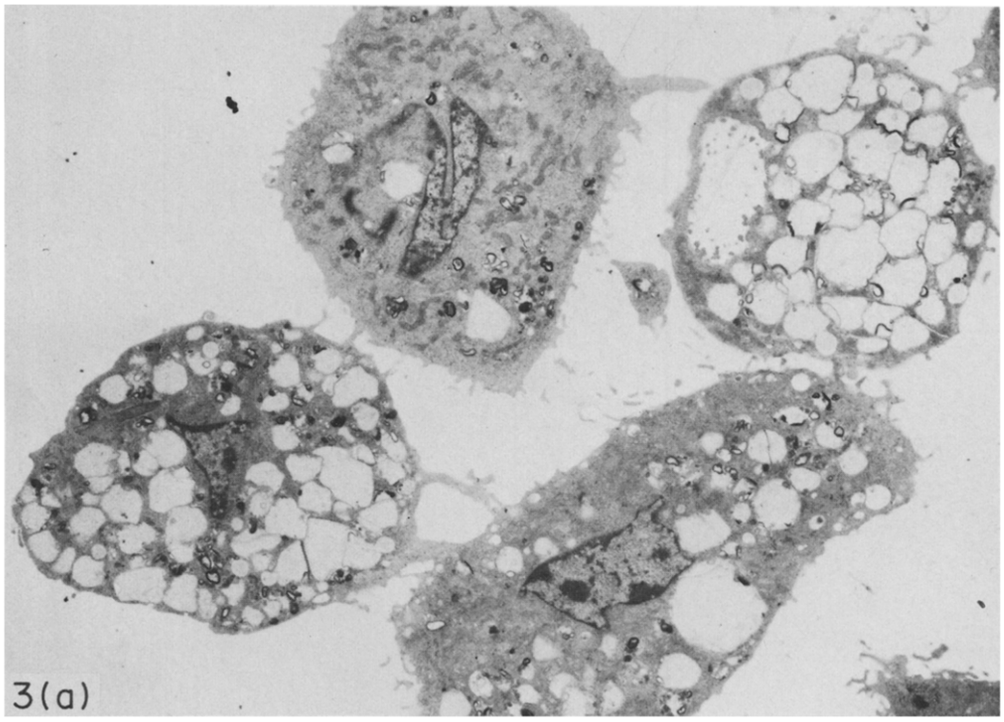


Fig. 2. *Phase contrast micrographs of cultured Mz-Sto-*1 *signet ring cancer cells, displaying abundant intracytoplasmic vacuoles. Magn.* × 240.



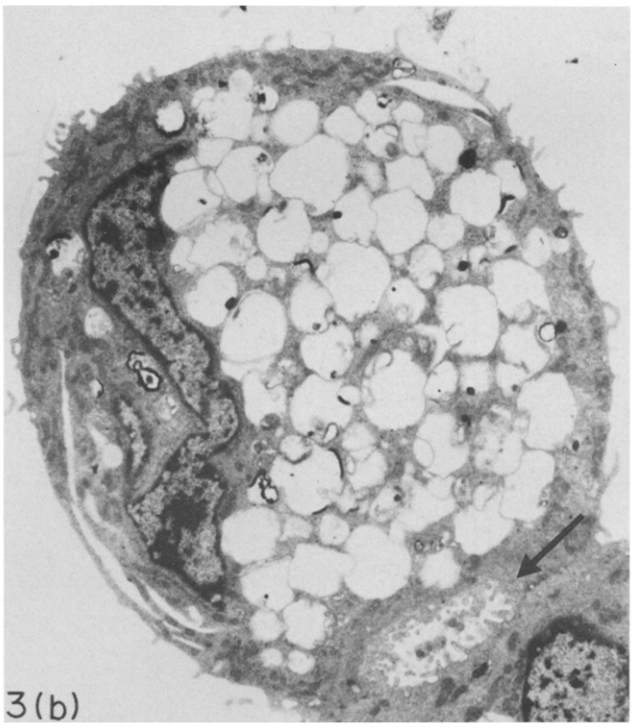


Fig. 3. (*a*) *Transmission of electron micrographs of Mz-Sto-*1 *cells, exhibiting membrane-bound vacuoles, which contain flocculent translucient material and push the nucleus to the periphery. Magn.* × 3040. (*b*) *Intercellular acinar spaces are marked* ↑ . *Magn.* × 5280.

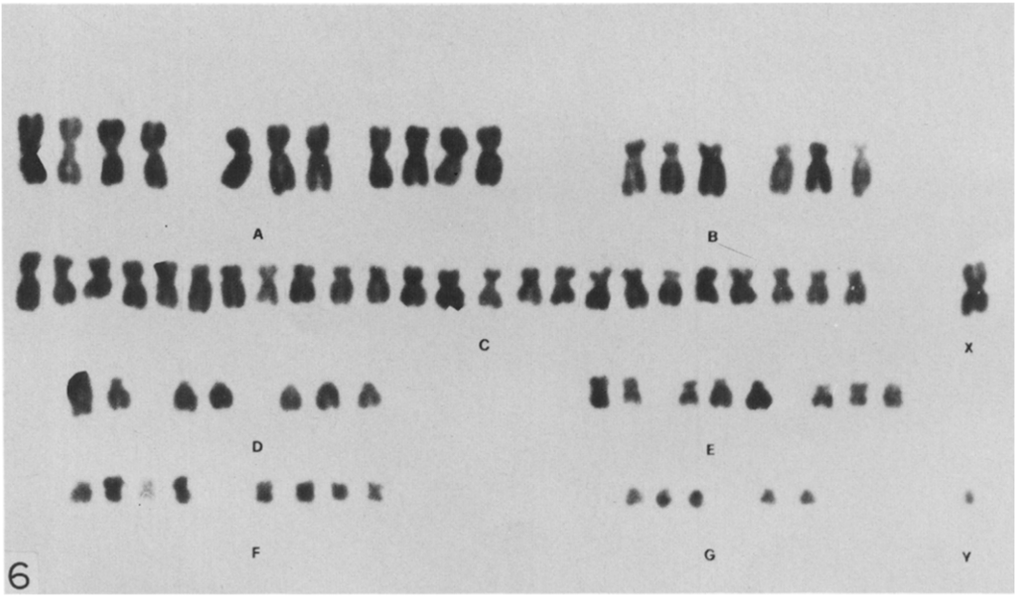


Fig. 6. Characteristic karyotype of Mz-Sto-1 cells.

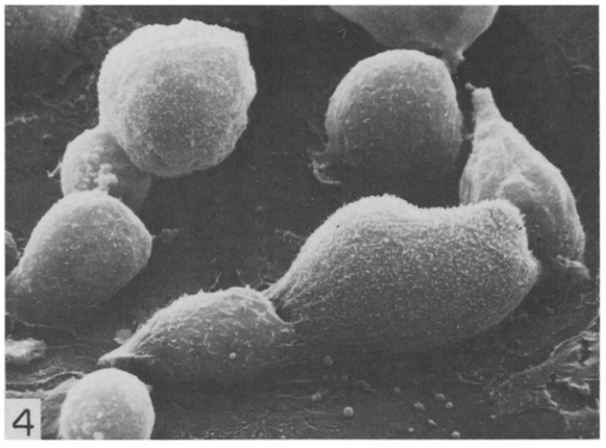


Fig. 4. Scanning electron micrographs of Mz-Sto-1 tumor cells showing microvilli on the cell surface. Magn. × 1000.

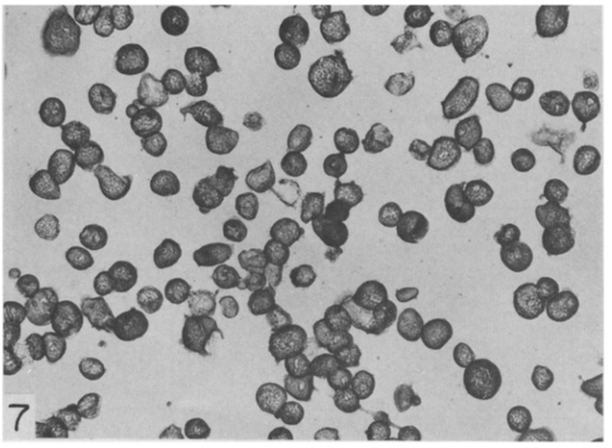


Fig. 7.

Serological tests, reagents and biochemical markers

Mz-Sto-1 cells were plated and cultured for 3 days. The culture medium (10 ml) was then replaced and the cell-free supernatants were collected 72 hr thereafter, to determine secreted products. A commercially available enzymatic test was used for *N*-acetyl neuraminic acid (NANA) (Boehringer Mannheim, W. Germany). In this assay the glycosidically bound sialic acid is hydrolyzed by neuraminidase to release free *N*-acetyl neuraminic acid. Alpha-1-fetoprotein (AFP) and carcinoembryonic antigen (CEA) were determined by ELISA-test kits (Abbott Laboratories, Chicago, IL, U.S.A.). Cell surface antigens of cultured cells were tested by an ELISA-test, using rabbit anti-mouse Ig-peroxidase, diluted 1 : 10 (DAKOPATTS, Copenhagen, Denmark). Two hundred living cells per well in culture medium were seeded into microtest plates (Falcon No. 3034) and incubated overnight at 37°C and 5% CO_2 atmosphere to allow cell attachment. Then cells were washed with PBS + 1% FBS gammaglobulin (gg)-free, 10 µl of test antibody was added and after an incubation at room temperature for 45 min, cells were washed again, and overlayed with rabbit anti-mouse Ig-peroxidase. As substrate for the immunoperoxidase test, freshly prepared 3-amino-9-ethyl-carbazol (Sigma Chemicals, St. Louis, U.S.A.), a red stain, was applied for 10 min. Afterwards, the plates were washed under running tap water for at least 5 min. Monoclonal antibodies (mAbs) were used at an antibody concentration of about 50 µg/ml. The following mAbs were purchased: W6/32 [21] to human HLA-determinants from Sera-lab, Crawley Down, Sussex, England; mAbs to human blood group antigens A, B, Lewis^{a,b} from Biotest Diagnotica, Frankfurt, W. Germany. MAb L 243 [22] was obtained from L.A. Lampson through the American Type Culture Collection (ATCC), Rockville, MD, U.S.A. Another mAb to monomorphic human Ia-determinants (691-13-17) was described in Reference [23] and the mAb to the transferrin-related molecule gp95 in [24, 25]. Antibodies against carcinoembryonic antigen (CEA) were kindly supplied by J.P. Mach [26].

RESULTS

Morphological criteria and growth kinetics of cultured Mz-Sto-1 gastric signet ring cancer cells

The primary tumor is classified as moderately well-differentiated adenocarcinoma with foci of signet ring cells (Fig. 1). Cultured Mz-Sto-1 gastric cancer cells, derived from tumorous ascites fluid, adhere to the tissue culture flask, grow preferentially in small islands and exhibit an epithelial cell-type morphology as depicted by phase contrast microscopy in Fig. 2. Most cells contain cytoplasmic vacuoles, sometimes of enormous size. Transmission

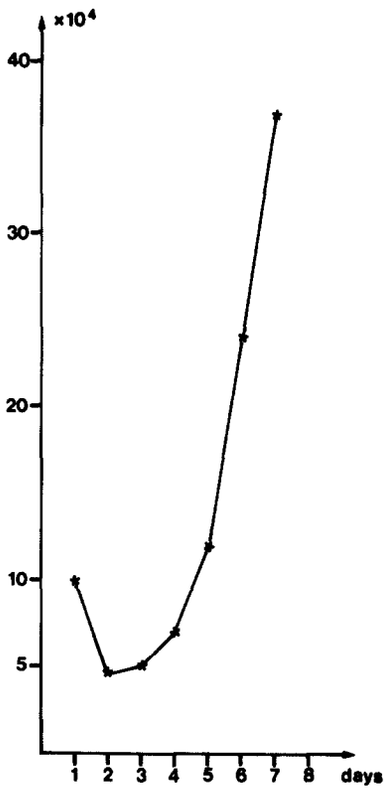


Fig. 5. Growth curve of Mz-Sto-1 cells.

electron micrographs reveal that the vacuoles are filled with flocculent translucent material (Fig. 3a), often pushing the nucleus to the periphery, as typical for signet ring cells (Fig. 3b). Some vacuoles are surrounded by membranes or display myelin figures at the rim. Furthermore, the tumor cells exhibit intercellular acinar spaces, characteristic for adenocarcinoma. By scanning electron microscopy the Mz-Sto-1 cells show different size and morphology with microvilli studding the cell surface (Fig. 4). Mz-Sto-1 cells (passage 22) double in 28 hr (see Fig. 5), if plated densely. Their plating efficiency amounts up to 50%. Up to now Mz-Sto-1 cells have been maintained for 35 continued passages in tissue culture. Mycoplasma were neither observed by the 33258 Hoechst stain nor by electron microscopy in Mz-Sto-1 cells. Growth of tumors in the back of NMRI nude mice was obtained in 1 out of 2 trials 4 weeks after inoculation of 2×10^7 cells. This mouse tumor was transferred to tissue culture again and the morphology of the growing tumor cells was comparable to the initial culture.

Chromosomal analysis

Chromosome analysis of Mz-Sto-1 cells (cell passage number 17, 18, 19, 20) reveals a narrow range of chromosome number. Sixteen out of 50 metasphases analyzed contain 72, 27 contain 73 and 7 metaphases 74 chromosomes. Sixteen microchromosomes are counted in all 50 metaphases. The karyotype shows a marked tendency to tetraploidy,

particularly in subgroup C, but no marker chromosomes. A representative example is depicted in Fig. 6.

Oncogene amplification in gastric cancer cell lines

Cellular DNA (10 μg) of Mz-Sto-1, MKN-28, MKN-45 gastric cancer cell lines, was digested with restriction enzyme EcoRI and hybridized under stringent conditions with a radioactive labeled probe containing *myc* exons 1, 2 and 3. Autoradiography showed a single band of comparable intensity at 13 kb in all 3 gastric cancer cell lines and in 10 μg of normal human placenta DNA and of peripheral lymphocytes from a healthy volunteer. As positive control, 10 μg DNA of the SK-BR-3 breast carcinoma cell line were used, which showed a strong positive signal, representing a 10-fold *c-myc* amplification [27]. These results indicate, that *c-myc*, which is entirely represented within the 13kb EcoRI DNA fragment is neither amplified nor underwent major rearrangement in these cell lines. To see if other oncogenes are amplified in Mz-Sto-1 cells, 1 μg nick-translated Mz-Sto-1 and SK-BR-3 DNA as positive control were hybridized to a Southern blot containing the following oncogene DNAs: *erbA*$_1$, *erbA*$_2$, *erbB*, *Ha-ras*, *Ki-ras*, *N-ras*, *myc*, *src*, *raf*, *myb*, *sis*, *rel*, *fgr*, *yes*, *fes*, *fms*, *fos*, *mos*, *abl*. No autoradiographic signals were detected with Mz-Sto-1 DNA. A positive signal, however, was seen with SK-BR-3 and *c-myc* DNA. Therefore, a more than 10-fold amplification of any of these oncogenes in Mz-Sto-1 can be excluded.

Antigenic markers of human gastric cancer cell lines

Two out of 3 established gastric cancer cell lines (Mz-Sto-1, MKN-28, MKN-45) produce carcinoembryonic antigen (CEA), but no alpha-1-fetoprotein. Mz-Sto-1 cells accumulate 150–250 ng/10^7 cells CEA, MKN-45 100 ng and MKN-28 no CEA in the cell-free culture supernatants after 3 days of culture. CEA-production of cultured Mz-Sto-1 cells corresponds to an increased CEA-value (16 ng/ml) in the serum of patient G at the day of taking his tumor cells. *N*-Acetylneuraminic acid is not elevated in the supernatant of cultured Mz-Sto-1 cells.

Furthermore, a number of cell surface antigens are identified on gastric cancer cell lines using monoclonal antibodies in an ELISA test, as shown in Table 1. All 3 cell lines express HLA, blood group A and Lewis antigens. As an example, the positive staining of Mz-Sto-1 cells with monoclonal anti-Lewis^a antibody is shown in Fig. 7. Mz-Sto-1 and MKN-45 cells react with 3 monoclonal antibodies (mAb 73, 35, 192) to CEA, MKN-28 cells do not. HLA–DR antigens are not found on all 3 gastric cancer cell lines, but on 6 different specimens of normal stomach epithelium. Monoclonal antibodies to melanoma-associated antigens (gp95, ganglioside G_{D3}), which were used as negative controls, are not expressed.

Table 1. Cell surface antigens of human gastric cancer lines

Antigens	Gastric cancer cell lines Mz-Sto-1	MKN-28	MKN-45
HLA (W6/32)	+	+	+
A blood group	+	+	+
B blood group	−	−	−
Lewis^a	+	+	−
Lewis^b	+	−	(+)
CEA (mAb 73, 35, 192)	+	−	+
gp95 (I12)	−	−	−
HLA–DR (L243, 13–17)	−	−	−

DISCUSSION

Gastric carcinomas are difficult to get established in permanent tissue culture and reports on human cancer cell lines are sparse. To our knowledge Mz-Sto-1 cells represent the first ultrastructurally defined signet ring carcinoma cells. The morphological characteristics of Mz-Sto-1 cells describe features for epithelial cells. Furthermore membrane-bound vacuoles, which push the nucleus to the cell periphery, and intercellular acinar spaces represent typical features for these *in vitro* cultured signet ring cell carcinomas. We observed the same ultrastructural characteristics in tissue sections of primary signet ring cell tumors (figure not shown).

The chromosomal analysis of Mz-Sto-1 tumor cells reveals a narrow range of chromosome number (between 72 and 74) and a marked tendency to tetraploidy, particularly in subgroup C. These chromosomal characteristics of Mz-Sto-1 cells resemble similar findings by Motoyama *et al.* [10] in the gastric cancer cell lines KATO II and III, which show cytological features, consistent with signet ring cancer cells.

In this preliminary screening no amplification of 19 different oncogenes, including *c-myc*, was found in Mz-Sto-1 cells. Recently, 2 reports on the amplification of *c-myc* in 2/11 [13] respectively 3/16 [14] gastric carcinomas had suggested that this amplification event could be more frequent in gastric carcinoma than in other tumors. Using the methods described, only amplification of oncogenes greater than 10 genome equivalents would have been detected. Therefore minor amplification of oncogene DNA may have been missed. In regard to *c-myc* no major rearrangement was observed in Mz-Sto-1 DNA in comparison to control DNA.

Up to now, very little is known about cell surface antigens of human gastric cancer cell lines. Here we demonstrate that they express HLA antigens,

blood group-related antigens and CEA. The expression of blood group and blood group-related antigens in human epithelial cancers is well known [28–31]. In Mz-Sto-1 cells, no incompatible blood group expression was found, because the patient's tumor and red blood cells carry A determinants. None of these 3 gastric cancer cell lines, however, reacts with 2 different monoclonal antibodies to HLA–DR, neither do several frozen tissue sections of gastric carcinoma (preliminary results, data not shown). In contrast, tissue samples of normal stomach epithelium stain very strongly with these 2 HLA–DR antibodies by immunohistochemical methods. The expression of immune response antigens in normal stomach epithelium is known [32]. Its loss in gastric cancer and the expression in premalignant lesions, however, remains to be further elucidated.

Although monoclonal antibodies to gastric cancer have been raised [33–35], the challenge still remains, to define new restricted markers for gastric carcinoma and to study the biology of these tumors.

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