Establishment and Characterization of an Amylase-Producing Human Myeloma Cell Line

By Hiromitsu Matsuzaki, Hiroyuki Hata, Motohiro Takeya, and Kiyoshi Takatsuki

Two stable lines of IgA-α-producing plasma cells (KHM-1A and KHM-1B) that were free of the Epstein-Barr virus were established from a patient with multiple myeloma complicated by hyperamylasemia. Surface marker studies of the two cell lines showed that the cells had no surface immunoglobulins but were positive for cytoplasmic immunoglobulins (IgA λ) and for HLA-DR and PCA-1. Secretion of IgA monoclonal immunoglobulin by the two lines was detected by a plaque-forming cell assay and by an enzyme-linked immunosorbent assay of culture media. KHM-1B cells also secreted α-amylase, but no such activity was detected in the culture-conditioned supernatant fluid of KHM-1A.

HYPERAMYLASEMIA resulting from ectopic production of amylase by tumor tissue was first described by Weiss et al. in 1951. Since then, several cases of hyperamylasemia have been reported, especially in pulmonary and ovarian cancers. However, only one amylase-producing cell line has been established, that being from gastric carcinoma. In addition, malignant plasma cells have also proved to be very difficult to establish, and only a little over ten human myeloma cell lines have been reported. In this study, we established two human myeloma cell lines, named KHM-1A and KHM-1B, from a patient with IgA-λ multiple myeloma. KHM-1A cells secreted only IgA, while KHM-1B cells secreted IgA and α-amylase. This is the first report, to the best of our knowledge, of an amylase-producing nonepithelial cell line.

MATERIALS AND METHODS

Case report. A 53-year-old man admitted to Kumamoto University Hospital in November 1985 was diagnosed as having IgA-λ multiple myeloma with hyperamylasemia. Iszyme analysis of serum and urine by electrophoresis revealed that the amylase was of the salivary type. Following improvement of the hyperamylasemia and the serum IgA level of treatment with melphalan and prednisolone, in May 1986 he developed a plasmacytoma on the left chest wall and plasma cell–induced pleuritis carcinomatosa. Amylase activity was markedly elevated in both the pleural fluid and the serum and was also detected in the supernatant fluid of cultured pleural effusion myeloma cells. The presence of IgA and amylase in the myeloma cells was demonstrated immunohistochemically. The patient died of pneumonia in November 1986. Autopsy revealed multiple myeloma, with widespread plasmacytomas in the lungs, liver, pancreas, testes, pleura, diaphragm, and peritoneum.

Cell culture. Heparinized pleural effusions from the patient were layered on the Ficoll-Conray (specific gravity, 1.078) and were centrifuged at 400 g for 30 minutes. The interphase cells, which consisted almost entirely of myeloma cells, were collected and seeded into culture plates at approximately 10^5 cells/mL after washing with complete medium. In primary culture and the early passages, RPMI 1640 medium containing 20% fetal calf serum (FCS) was used, but when the cells began to grow steadily, the medium was changed to RPMI 1640 containing 10% FCS. The cells were maintained throughout under 5% CO2 in humidified air at 37°C.

Immunohistochemical analyses of amylase and immunoglobulins. The localization of amylase and IgA within the cultured cells was demonstrated immunohistochemically by an indirect immunoperoxidase method using rabbit antibodies against human salivary-type amylase and IgA (Dakopatts, Copenhagen). Control slides were incubated the same way, with nonimmunized rabbit serum as the first antibody or by omitting the first antibody.

Immunoelectron microscopy. Myeloma cells were fixed with 2% periodate-lysine-paraformaldehyde fixative for 60 minutes. After washing with phosphate-buffered saline, the cell pellets were frozen in ornithine carbamyl transferase compound, and frozen sections were prepared. The sections were then treated with rabbit antiymalase antibody (F[ab']2) followed by incubation with the peroxidase-conjugated antirabbit Ig (F[ab']2) (Amersham Corp., Buckinghamshire, England). After visualization of the peroxidase activity with 3,3′-diaminobenzidine as the substrate, the cells were postfixed with 1% osmium tetroxide, dehydrated, and embedded in epoxy resin as described previously. Control experiments were performed in the same way as the light microscopic immunohistochemistry.

Immunologic marker studies. Measurements of the following cell surface markers were carried out: HLA-DR, CD2, CD10, CD20, PCA-1 (Becton Dickinson, Mountain View, CA), and surface immunoglobulins (sIg). Cytoplasmic immunoglobulins were stained with fluorescein-conjugated antiimmunoglobulin antisera for one hour after cell fixation.

Assay for amylase in the culture media. Amylase activity in the culture media was measured enzymatically by the use of a soluble substrate, p-nitrophenyl maltoside, and a coupled enzymatic indicator reaction involving α-glucosidase (Boehringer Mannheim Biochemicals, Indianapolis).

Assay for plaque-forming cells. Antibody secretion was measured by a reverse hemolytic plaque assay as described previously.

Enzyme-linked immunosorbent assay for IgA in the culture media. Goat antibody against human IgA (heavy chain specific; Sigma Chemical Co., St. Louis) was adjusted to a concentration of 50 μg/mL in carbonate buffer (pH 9.6), and 100 μL was incubated overnight at 4°C in each well of 96-well microtiter plates. After 100 μL of 2% bovine serum albumin (BSA) in Tris buffer (pH 7.4) had been added and the incubation continued for an additional two hours, the wells were washed three times with Tris buffer containing 0.2% BSA (Tris-BSA). This was followed by the addition of 100 μL of either culture supernatant fluid or control IgA to each well.
and by incubation at 4°C for two hours. After the wells had been washed three times with Tris-BSA, 100 μL of a 1,000-fold dilution of alkaline phosphatase-conjugated antihuman IgA (Sigma) was added and the wells incubated for an additional two hours at 4°C. The wells were then washed as described earlier, and 100 μL of phosphatase substrate (Sigma) was added to each. Absorbance at 405 nm was measured by using a Titertek Multiskan spectrophotometer (Lierbyen, Norway).

RESULTS

**Morphology of cultured cells.** The myeloma cells were collected and cultured from the patient’s pleural effusions at two different times. At early passage, the two types of cells obtained, called KHM-1A and KHM-1B, floated in the culture medium almost as single-cell suspensions. At a later passage, however, their characteristics gradually diverged. KHM-1A cells tended to attach singly to the plastic substrate, whereas KHM-1B cells formed soft clusters that continued to float. In Wright-Giemsa-stained smears, both KHM-1A (Fig 1A) and KHM-1B (Fig 1B) cells were morphologically similar to typical plasmablasts, having nuclei containing nucleoli and having basophilic cytoplasm. However, in KHM-1A cultures multinucleated plasmablasts abounded (Fig 1A). Immunoperoxidase studies using anti-amylase antibody showed that most KHM-1B cells were positive (Fig 2B), whereas practically no KHM-1A cells were positive (Fig 2A).

**Immunoelectron microscopy.** Electron microscopy demonstrated that both the KHM-1A and KHM-1B cells possessed well-developed rough endoplasmic reticulum. Immunoelectron microscopic examination showed that most KHM-1B cells were stained with anti-amylase antibody (Fig 3), whereas only a few KHM-1A cells were positive. The reaction products were observed intracytoplasmically, especially coincident with the well-developed endoplasmic reticulum. No secretory granules were observed.

**Surface markers.** Both KHM-1A and KHM-1B cells were positive for HLA-DR and PCA-1 but negative for CD2 (OKT11), CD10 (CALLA), and CD20 (Leu-16). Living cells were analyzed with the fluorescein-conjugated anti-immunoglobulin antisera for IgGs and were found to be unstainable. With fixed cells, α chain and λ immunoglobulins were detected in the cytoplasm.

**Plaque-forming cell assay and enzyme-linked immunosorbent assay for IgA in the culture media.** The plaque-forming cell (PFC) assay detected secretion of IgA by both the KHM-1A and KHM-1B cells (Table 1). Quantitative enzyme-linked immunosorbent assay

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**Fig 1.** Cytologic appearance of KHM-1A (A) and KHM-1B (B) cells. KHM-1A cells contained many multinucleated plasma cells (Wright-Giemsa stain).

**Fig 2.** Immunoperoxidase staining of KHM-1A (A) and KHM-1B (B) cells for α-amylase. No KHM-1A cells were positive, while many KHM-1B cells were strongly positive (counterstained with hematoxylin; original magnification ×450).
Fig 5. Cell number and amylase secretion. Cell number (○) and amylase activity (●, △) in the culture medium were assayed daily for 12 days. No medium changes were made during the experiment.

Fig 4. Accumulation curve for IgA secretion per 5 × 10⁴ KHM-1A and KHM-1B cells per milliliter. IgA levels were assayed daily for six days without medium changes.

with time of amylase in the culture medium. The doubling time of KHM-1A cells was about five days and that of KHM-1B about two days (Fig 5). An almost linear increase in amylase activity in the culture medium of KHM-1B cells was observed until day 12 of cultivation, but no amylase activity was detected in the culture medium of KHM-1A cells (Fig 5).

DISCUSSION

Despite the large number of cell lines in continuous culture that were derived from patients with myeloma, only a limited

<table>
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<th>Table 1. Immunoglobulin-Secreting KHM-1 Cells</th>
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<th>μ</th>
<th>κ</th>
<th>λ</th>
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<td>101,687</td>
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<td>0</td>
<td>0</td>
<td>623,458</td>
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*PFCs/10⁶ cells.
number are actually myeloma cell lines.15-24 The majority of lines are said to be derived from nonneoplastic Epstein-Barr virus (EBV)-infected B lymphocytes that grew out from the tumor tissue.25 In this report we describe the establishment of two EBV-negative subclonal myeloma cell lines. Routine light microscopy, immunohistochemistry, and electron microscopy indicated that they had plasma cell morphologies that were retained in long-term culture. Production and secretion of monoclonal IgA lambda immunoglobulin, the same serum isotype as the patient’s monoclonal protein, by both myeloma cell lines was observed by immunoperoxidase staining, PFC assay, and ELISA. In addition, KHM-1B cells were found to produce and secrete α-amylase. As already mentioned, many cases of ectopic amylase production by tumors have been reported, especially in pulmonary and ovarian cancers, but to our knowledge, this is the first case of an amylase-producing nonneoplastic cancer. In addition, KHM-1B is only the second reported amylase-producing cell line, the first being a gastric cancer–derived cell line, KMK-2, established by Nomura et al14 at Kumamoto University Medical School.

Production of a range of hormones by cancer cells has been recognized and interpreted as expression of an ectopic phenotype, and their production of α-amylase may represent another example of this phenomenon. However, at least two other interpretations are possible for these rare cancer phenotypes. Production of amylase by the cancer cells may be the result of overproduction of a small quantity of normally existing protein since amylase has been demonstrated in normal lungs,29,30 liver,31,32 oviducts,33 and even leukocytes34 as well as in the salivary glands and pancreas. Normal plasma cells may also contain amylase. However, the possibilities remain that these amylase-containing tissues take up salivary or pancreatic enzyme from the serum or the tissues and, when examined, have been contaminated with serum.

The other possible interpretation is that the cancer-associated amylase is a fetal enzyme. An analogous situation can be seen in the production of alkaline phosphatase by cancer cells; alkaline phosphatase isoenzymes possibly have counterparts in normally developing embryonal tissues.35 Since fundamental studies of α-amylase in embryonal tissues have not been undertaken, this interpretation cannot be assessed at present.

Recently, Matsubara et al36-38 determined the nucleotide sequences of both salivary and pancreatic human α-amylase cDNAs. They reported that in the coding region the nucleotide sequence homology was 96% and that the predicted amino acid sequences were 94% homologous. They kindly gave us a sample of amylase cDNA. The amylase genes of KHM-1A and KHM-1B will be analyzed by using it.

REFERENCES

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