Localization and Characterization of Anionic Sites in Extramammary Paget's Disease with Cationic Colloidal Gold

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Extramammary Paget's disease is a slow-growing malignant disease occurring on the anogenital area and rarely on the axilla. Recent immunohistochemical studies have shown that Paget cells differentiate to secretory cells of sweat glands. However little is known about the expression of glycosaminoglycan in Paget cells and its relation to the differentiation of sweat glands. Therefore we studied the light and electron microscopic localization of anionic sites stained with cationic gold using post-embedding method. We also studied the digestibility of anionic sites with enzymes such as neuraminidase, chondroitinase ABC, and heparitinase, because anionic sites in normal eccrine and apocrine sweat glands show different susceptibility to these enzymes. Cationic gold stained 19.6±3.0% of Paget cells at pH 2.0, although most of these anionic sites were not stained at pH 7.4. Anionic sites in Paget cells were completely digested with neuraminidase, however chondroitinase ABC or heparitinase did not digest them. Enzyme susceptibility and paradoxical pH-dependency of anionic sites in Paget cells were the same as those of apocrine secretory cells and completely different from those of eccrine secretory cells, ductal cells of sweat glands or epidermal keratinocytes. Therefore, the expression of glycosaminoglycan labeled with cationic gold indicates that Paget cells differentiate into secretory cells of apocrine sweat gland.

Key words: Apocrine sweat gland, Epidermis, Neuraminidase, Sialic acid

I. Introduction

Extramammary Paget's disease is a poorly understood malignant disease on the anogenital area and rarely on the axilla [4, 11]. Paget cells are large cells with large oval nuclei and abundant pale vacuolated cytoplasm [7, 9]. Although the cell of origin is unclear, most believe Paget cells are derived from pluripotent cells in the epidermis and differentiate into sweat gland cells [9]. However, it has been controversial whether Paget cells differentiate into the cells in eccrine or in apocrine sweat glands. Recent immunohistochemical studies suggest that Paget cells differentiate to apocrine sweat glands [9].

The human body has two types of sweat glands, eccrine and apocrine sweat glands. These sweat glands are different in function and in structure [13, 19]. Eccrine sweat glands cover almost the entire body surface. The main function of the eccrine sweat gland is the control of body temperature. An eccrine sweat gland is a simple tubular structure that consists of secretory and ductal portions. The secretory portion of eccrine sweat glands produces nearly isotonic primary sweat. The ductal portion reabsorbs Na from the primary sweat, then hypotonic sweat is secreted on the surface of skin [19]. The apocrine sweat gland is a large sweat gland and is thought to be a remnant of an odorous organ of animals. It is also a simple tubular structure that consists of secretory and ductal portions. Ducts of apocrine sweat glands open to hair follicles, although ducts of eccrine sweat glands open directly to the surface of the skin. Apocrine sweat glands are distributed mainly on the axillary and genital areas [13, 19].

It is sometimes difficult to determine whether sweat gland tumors are differentiating into the cells of eccrine or apocrine sweat glands because there are few clear-cut histological markers to differentiate these two types of sweat glands. Past special staining for mucin and enzyme digestion studies have shown that both eccrine and apo-
crine sweat glands express sialic acid [1, 6]. Thus these staining methods cannot be used to investigate the differentiation of extramammary Paget’s disease. A recent study from our laboratory showed that cationic gold stained anionic sites in eccrine and apocrine sweat glands in a different manner [16]. Enzyme digestion before the staining with cationic gold revealed that eccrine sweat glands express chondroitin sulfate and apocrine sweat glands express sialic acid. Therefore, we studied the localization and enzyme digestibility of anionic sites in Paget cells using cationic gold.

II. Materials and Methods

Tissues

Six cases of extramammary Paget’s disease (3 male and 3 female patients) without underlying carcinomas and without distant carcinomas were included in the study. Tissues were processed as reported previously [16, 17]. The methods of fixation and embedding are essentially the same as widely employed for post-embedding immuno-cytochemistry [15, 25]. Small pieces of tissues were cut from surgically excised lesions of genital Paget’s disease, and these tissues were fixed in a mixture of 3% para-formaldehyde and 0.5% glutaraldehyde in PBS (0.01 M phosphate buffer, pH 7.4) for 1 hr at room temperature. After washing in PBS, aldehyde residue was quenched by incubating the specimens in 50 mM NH₄Cl in PBS for 30 min. The specimens were stored in PBS at 4°C overnight. Fixed specimens were dehydrated in the graded series of ethanol then embedded in Lowicryl K4M. The plastic was cured with ultraviolet rays at −20°C using TUV-200 (Dosaka EM, Kyoto, Japan).

Light microscopic staining

For light microscopic observation, one μm semi-thin sections were cut with a glass knife and mounted on poly-L-lysine coated slide glasses. A section on a slide glass was preincubated with a drop of PBS (pH 7.4) containing 0.1% BSA for 10 min. The sections were then stained with cationic colloidal gold conjugated with poly-L-lysine (1:20) in PBS (pH 2.0 or pH 7.4) containing 0.2% BSA for 1 hr at room temperature. The sections were washed with PBS (pH 7.4) containing 0.1% BSA (10 min × 6), and distilled water (2 min × 2). Enzyme digestion before the staining with cationic gold revealed that eccrine sweat glands express chondroitin sulfate and apocrine sweat glands express sialic acid. Consequently, sections were stained with cationic colloidal gold as described previously.

Enzyme digestion

Enzyme digestion study of anionic sites was carried out using chondroitinase ABC, heparitinase and neuraminidase before staining with cationic gold. Each enzyme was prepared as follows. Chondroitinase ABC was dissolved in 0.1 M Tris-HCl buffer supplemented with 0.03 M sodium acetate, pH 8.0 at the concentration of 2 U/ml. Heparitinase was dissolved in 0.1 M Na-acetate buffer, pH 7.0 at a concentration of 0.1 U/ml. Neuraminidase was dissolved in 0.1 M Na-acetate buffer, pH 5.4 containing 0.01 M CaCl₂ at a concentration of 4 U/ml. Grids were incubated on the droplets of each enzyme solution for 2 hr at 37°C in humid chambers. After washing on droplets of PBS, grids were stained for anionic sites as previously described. Control grids were incubated on the droplets of each buffer without enzymes.

EGTA treatment

EGTA (ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid) treatment was performed as follows. EGTA (ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid) treatment was performed as follows. Sections on glass slides for light microscopy and on grids for electron microscopy were incubated with 0.2 mM EGTA for 20 min at room temperature. After that, sections were washed in three changes of deionized water. Consequently, sections were stained with cationic colloidal gold as described previously.

Chemicals

Cationic colloidal gold and a silver enhancing kit was purchased from BioCell Research Laboratory (Cardiff, UK). Lowicryl K4M was obtained from Polyscience (Warrington, PA). Neuraminidase from Clostridium perfringens was purchased from Sigma (St Louis, MO). Chondroitinase ABC protease free from Proteus vulgaris, heparitinase from Flavobacterium heparinum were purchased from Seikagaku Corporation (Tokyo, Japan).

III. Results

Selective and specific localizations of anionic sites in normal sweat glands were reported in our previous electron microscopic study [16]. Since our preliminary experiments showed heterogeneity of Paget cells in staining with cationic gold, we employed additional light microscopic observation to study many cells in the same section. First we confirmed the validity of light microscopic staining using normal sweat glands. Light
microscopic staining of normal apocrine sweat glands with cationic gold was consistent with previous electron microscopic observation. Luminal cell membranes of apocrine secretory cells were intensely labeled with cationic gold at pH 2.0 (Fig. 1a). Alteration of pH showed a prominent effect on the light microscopic distribution of anionic sites in apocrine sweat glands. Luminal cell membranes of apocrine secretory cells that were stained at pH 2.0 were not stained at pH 7.4 as previously reported with electron microscopy (Fig. 1b). Light microscopic enzyme digestion study of these anionic sites showed the same digestibility as observed with electron microscopy. Neuraminidase completely digested anionic sites on the luminal membranes of apocrine secretory cells however other enzymes did not digest them (data not shown). It was difficult for light microscopes to discriminate labeled basolateral cell membranes of clear secretory cells of eccrine sweat glands from surrounding connective tissue that was also labeled with cationic gold (data not shown). Therefore both light and electron microscopic observations were carried out in our study of extramammary Paget’s disease stained with cationic gold.

Fig. 1. Light microscopic staining of apocrine sweat glands with cationic gold. (a) Luminal cell membranes of secretory cells were strongly stained at pH 2.0. Original magnification × 130. Bar = 100 μm. (b) Cytoplasm and nuclei were diffusely stained at pH 7.4. Original magnification × 260. Bar = 50 μm.

Fig. 2. Serial sections of extramammary Paget’s disease were stained with cationic gold for light microscopic observation. Dotted lines indicate the junction of epidermis and dermis. Paget’s cells were located in the basal layer and the lower epidermis as a single cell or as a group of a few cells. (a) Some Paget’s cells (arrows) in the epidermis were strongly stained at pH 2.0. (b) All Paget’s cells and epidermal keratinocytes showed diffuse staining at pH 7.4. (c) The section was preincubated with neuraminidase before the staining with cationic gold at pH 2.0. Neuraminidase completely digested positive staining observed without pretreatment. Original magnification × 130. Bar = 100 μm.
Paget cells were distinguished from normal keratinocytes on the Lowicryl K4M-embedded sections because of large irregularly shaped nuclei and abundant cytoplasm. Light microscopic staining for anionic sites at pH 2.0 demonstrated positive black granules on the cytoplasm (Fig. 2a) of 19.6±3.0% (mean±standard error, n=6) of Paget cells. At pH 7.4, the cytoplasm and nucleus of every Paget cell and surrounding keratinocytes showed diffuse staining. However, strongly positive cells observed at pH 2.0 did not show a stronger reaction than

![Fig. 3. Electron microscopic staining of a Paget cell with cationic gold at pH 2.0. Cytoplasmic vacuoles were stained with cationic gold. Original magnification ×25,000. Bar=0.5 μm.](image3)

![Fig. 4. Electron microscopic staining of a Paget cell with cationic gold at pH 7.4. Cytoplasm and nuclei were diffusely stained. However, most cytoplasmic vacuoles were not stained. Original magnification ×25,000. Bar=0.5 μm.](image4)
other Paget cells at pH 7.4 (Fig. 2b). Positive staining of Paget cells observed at pH 2.0 was abolished by the preincubation with neuraminidase (Fig. 2c), however preincubations with chondroitinase ABC or heparitinase did not alter the staining of these anionic sites (data not shown).

Electron microscopic observation showed that some Paget cells had electron lucent vacuoles in the cytoplasm. These cytoplasmic vacuoles were not membrane-delimited and did not show any internal structures. Cationic gold labeled these cytoplasmic vacuoles at pH 2.0 (Fig. 3), however most of these vacuoles were not labeled at pH 7.4 (Fig. 4). Preincubation with neuraminidase abolished labeling with cationic gold in Paget cells at pH 2.0 (Fig. 5). Preincubations with chondroitinase ABC or heparitinase did not abolish these anionic sites (data not shown). It is therefore concluded that these Paget cells correspond to the cells labeled with cationic gold shown with light microscope.

Previous report showed that calcium blocks anionic sites in dark cell granules of eccrine secretory cells [16]. Therefore we tested whether calcium blocks anionic sites in Paget cells. Preincubation of the semi-thin sections or ultrathin sections with EGTA did not alter the labeling with cationic gold at pH 2.0.

IV. Discussion

Extramammary Paget’s disease is a slow-growing malignant disease of aged people [4, 11]. It occurs as an eczematous or a fungus infection-like patch on the ano-genital area and very rarely on the axilla. Later, it may metastasize and may result in death [3]. About 20% of cases are associated with an extracutaneous carcinoma, and carcinoma of urogenital tract, rectum, and breast [4]. In these cases, Paget cells in the epidermis could be the direct extension of underlying carcinoma, epidermotropic metastasis, multifocal carcinoma, or pure coincidence. We studied extramammary Paget’s disease without underlying carcinomas and without any distant carcinomas in this report. Therefore tumor cells must have originated from the epidermal cells.

Histology of extramammary Paget’s disease shows large Paget cells with clear cytoplasm and atypical nuclei in the epidermis [7, 9]. It has been suggested that Paget cells are derived from pluripotent cells in the epidermis and differentiate into sweat gland cells. Paget cells contain glycosaminoglycan [4, 7] and express carcinoembryonic antigen [8], epithelial membrane antigen [5], and keratin intermediate filaments characteristic to secretory portions of sweat glands [21]. Gross cystic disease fluid protein was once thought to be a specific marker of apocrine sweat glands [10], and the presence of this protein in Paget cells supported the idea that Paget cells differentiated into apocrine sweat glands [10]. However later studies showed that these gross cystic disease fluid proteins exist in both eccrine and apocrine sweat glands [12]. Immunohistochemical expression of Ca 15-3 and Ka-93, and the
negative staining of Ca 19-9 and CD44 in extramammary Paget disease favored the idea that Paget cells originated from apocrine secretory cells [23]. Antiserum raised against formalin-fixed human milk fat globule glycoprotein stained Paget cells in extramammary Paget’s disease [8] and apocrine sweat glands but it did not stain eccrine sweat glands or sweat ducts [2]. Taken together, immunohistochemical studies support the notion that Paget cells differentiate into apocrine sweat glands.

Presence of mucin in Paget cells [4, 7] supported that these cells differentiate to sweat gland cells. Special staining for mucin using alcian blue and colloidal iron, and enzyme digestion studies have shown that both eccrine and apocrine sweat glands express sialomucin [1, 6]. Therefore demonstration of sialomucin by conventional mucin staining [4, 18] does not determine the direction of differentiation in Paget cells. Positively charged colloidal gold stains anionic sites on the sections embedded in the hydrophilic resins [14, 20, 24]. At pH 2.0, cationic gold labels sialic acid and sulfated glycosaminoglycans such as chondroitin sulfate, dermatan sulfate, and heparan sulfate [14, 22]. We have previously reported that eccrine and apocrine sweat glands showed different distribution of anionic sites and different susceptibility to enzyme digestion [16]. Secretory cells of eccrine and apocrine sweat glands were labeled with cationic gold at pH 2.0, however ductal cells or myoepithelial cells were not labeled. Eccrine sweat glands expressed anionic sites on basal cell membranes of clear secretory cells and on the submembranous vesicles of secretory cells. Neuraminidase digested these anionic sites. Therefore sialic acid was expressed exclusively in apocrine sweat glands, not in eccrine sweat glands. However, conventional mucin staining showed that both eccrine and apocrine sweat glands expressed sialic acid [1, 6]. Thus the difference of mucin expression between eccrine and apocrine sweat glands can be demonstrated with cationic gold method but not with conventional mucin staining such as alcian blue and colloidal iron.

In this study, light microscopic observation of EMPD showed that cationic gold intensely labeled about 20% of Paget cells at pH 2.0, not at pH 7.4. Neuraminidase completely digested these anionic sites, however chondroitinase ABC or heparitinase did not digest them. These results indicate that some Paget cells contain sialic acid that expresses anionic charge at pH 2.0, however not at pH 7.4. Electron microscopic observation demonstrated that cationic gold particles were localized on the vacuoles in the cytoplasm. These vacuoles were not delimited with membranes and they did not have any internal structures. The staining with cationic gold on these vacuoles were abolished by the preincubation with neuraminidase, not with other enzymes. Therefore these vacuoles contain sialic acid. The staining on these vacuoles with cationic gold was observed at pH 2.0, not at pH 7.4. These paradoxical pH-dependencies observed with both light and electron microscopies were not explained by charge theory alone. An effect of low pH might be to alter the structural configuration of sialoglycoprotein, thus rendering charge sites available for staining using cationic colloidal gold as proposed in apocrine sweat glands [16].

Staining with cationic gold showed that some Paget cells possessed anionic sites characteristic to the secretory cells of normal apocrine sweat glands. Previous study showed that epidermal keratinocytes express chondroitin sulfate and heparan sulfate, however they do not express sialic acid [17]. Therefore our data indicate that Paget cells stained with cationic gold at pH 2.0, expressed the glycosaminoglycan found in secretory cells of apocrine sweat glands but not in eccrine sweat glands or in normal epidermal keratinocytes.

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VI. References

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