Expression patterns of CD66a and CD117 in the mouse submandibular gland

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ABSTRACT

The epithelial tissue of the salivary gland consists of the acinar and ductal parts, the latter of which is further divided into the intercalated, striated and excretory duct segments and is the residential site for salivary stem/progenitor cells. In the present study, the expression patterns of two cell surface molecules, CD66a and CD117, were investigated in the adult mouse submandibular glands (SMG) by immunofluorescence microscopy. Combinations of the two molecules differentially marked several types of SMG epithelial cells, including acinar cells (CD66a-intense, CD117-negative), intercalated duct cells (CD66a-intense, CD117-positive), a subset of the striated and excretory duct cells (CD66a-weak, CD117-positive). Most of the CD117-positive ductal cells were negative for cytokeratin 5 and overlapped with the NKCC1-expressing cells. The CD117- and keratin 5-positive cells resided only in the excretory duct were suggested to correspond to the recently identified salivary stem cells. CD66a and CD117 may be useful markers to isolate several cell types consisting of SMG epithelium and to analyze their molecular and cellular nature. Our data also suggest that CD117-expressing epithelial cells of the gland include at least two distinct populations of the stem/progenitor cells.

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Introduction

The salivary glands consist of acinar and ductal parts, the latter of which are further segmented into the intercalated, striated, and excretory duct regions. Saliva is secreted by acinar cells and modified during the passage through ducts to the oral cavity (Lee et al., 2012; Zhang et al., 2013). Severe decrease in saliva secretion under certain conditions, including Sjögren syndrome and radiotherapy for head and neck cancer, causes xerostomia (dry mouth) and severe dental caries and oral disorders (Burlage et al., 2001; Lee and Le, 2008; Vissink et al., 2010). No satisfactory treatment for salivary gland hypofunction is currently available. An approach that offers the possibility of restoring the damaged glands is stem cell therapy (Coppes and Stokman, 2011). In order to develop stem cell therapy, an understanding at the molecular and cellular levels of properties of undifferentiated stem/progenitor cells as well as differentiated cells of salivary glands and of mechanisms of cell differentiation is required.

Stem/progenitor cells of the salivary gland have been demonstrated to reside in the ducts. Duct ligation of the salivary gland causes atrophy with loss of acinar cells and, after the removal of the ligation the remaining ductal cells proliferate and regenerate functional acini (Takahashi et al., 1998). Cell labeling experiments also suggested the location of stem/progenitor cells in the intercalated ducts (Denny and Denny, 1999; Man et al., 2001; Kimoto et al., 2008). Direct evidence for the existence of the progenitor cell population in the ducts of the gland was obtained by lineage tracing of cells expressing Ascl3, a transcription factor specifically expressed by a subset of the ductal cells (Yoshida et al., 2001; Bullard et al., 2008). Ascl3-expressing ductal cells had the ability to differentiate into both acinar and ductal cell types; however, they had little self-renewal activity, suggesting that they are progenitor but not stem cells (Bullard et al., 2008; Arany et al., 2011; Rugel-Stahl et al., 2012).

Recently, cell surface molecules CD117 (also referred to as c-Kit) and Sca-1, which are expressed by ductal cells in salivary glands, have been used to isolate putative stem/progenitor cells from the adult mouse SMG (Hisatomi et al., 2004; Lombaert et al., 2008; Feng et al., 2009; Nanduri et al., 2013; Xiao et al., 2014). The existence of cells with stem/progenitor cell activities in the CD117-expressing population was demonstrated by the in vitro sphere formation and...
in vivo transplantation assays (Lombaert et al., 2008, 2013; Nanduri et al., 2013). A small fraction of cultured SMG cells proliferated to form spheres with differentiated acinar and ductal cells and, following the transplantation of CD117-expressing cells isolated from the spheres into irradiated mouse SMGs, the saliva secretion of the mice was partially restored. More recently, it was furthermore demonstrated that, of the different cells isolated from the mouse SMG, the Lin−CD24−CD117+Sca1+ population exhibited the highest activity for proliferation, self-renewal and differentiation in vitro and in vivo (Xiao et al., 2014). It is far less understood, however, how salivary stem/progenitor cells identified so far relate to each other.

In the present study, we analyzed in detail the expression patterns of CD117 and CD66a in the adult mouse SMG, comparing them with those of several marker molecules. CD66a, also known as CEACAM1a, a member of the Ig superfamily of cell adhesion molecules, is expressed in various epithelial tissues including developing mouse SMG (Horst et al., 2006; Leung et al., 2006; Yokoyama et al., 2007; Mima et al., 2013) and has been used to isolate living cells (Gu et al., 2010). Our present data indicate that several epithelial cell types of adult mouse SMG, including acinar and ductal cells, are differentially marked by combinations of CD117 and CD66a, and provide immunohistochemical evidence for the heterogeneity of CD117-expressing cells.

Materials and methods

Animals and tissues

Eight-week-old virgin mice (ddY strain) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). The SMGs were dissected in Hanks’ balanced salt solution (HBSS) (Sigma-Aldrich, St. Louis, MO, USA), followed by the removal of the sublingual glands and attached fat tissues. SMGs were used for preparation of tissue sections and single cells. For preparation of tissue sections, SMGs were embedded in O.C.T. compounds (Sakura Finetek Japan, Tokyo, Japan), frozen in liquid nitrogen, and kept at −80 °C until use. Frozen sections (7 μm thick) were cut. All animal experiments were performed according to the Ethical Committee on Animal Testing of Osaka Dental University.

Dissociated cell preparation

Freshly prepared SMGs were minced on ice in HBSS with fine forceps and then digested with collagenase/hyaluronidase solution (StemCell Technologies, Vancouver, Canada) in DMEM/F12 containing Glutamax (GIBCO, Carlsbad, CA, USA) supplemented with 2% heat-inactivated fetal bovine serum (FBS) at 37 °C for 1 h using a rotator (30 rpm). The digested samples were washed with HBSS supplemented with 2 mM EDTA (HE) and centrifuged at 400 rpm for 1 min, which resulted in the enrichment of epithelial organoids in the pellet. The organoids were further digested with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 4 °C and then with dispase (5 mg/ml; StemCell Technologies) and DNase (200 μg/ml; StemCell Technologies) for 5 min. The digested cells were centrifuged and resuspended in HE supplemented with 2% FBS (HES), and then gently passed through 23-gauge syringes followed by filtration through 40 μm cell strainers (BD Biosciences). Ten SMGs yielded 1.0–1.5 × 10^7 cells. For immunostaining, single cells were spun down onto glass slides using a Cytospin (ThermoFisher, Waltham, MA, USA) at 350 rpm and air-dried.

Immunofluorescence microscopy

Tissue sections and single cells were fixed in cold methanol for 10 min at −20 °C. In some cases, tissue sections were incubated in

![Fig. 1. (a, b) Tissue sections of the adult mouse SMG were stained for CD117 and either CLD4 or AQP5. CD117 expression is seen in CLD4-positive ducts but not AQP5-positive acini. CD117-expressing cells cluster at the CLD4-positive region (arrows) adjacent to the striated ducts and are sparsely distributed in the striated ducts. (c, d) Dissociated cells prepared from the adult mouse SMG were stained for CD117 and either CLD4 or AQP5. CD117-expressing cells are positive for CLD4 but not AQP5. Nuclei are counterstained with DAPI. DIC, differential interference contrast microscopy. Scale bars = 25 μm.](image-url)
Fig. 2. (a, b) Tissue sections of the adult mouse SMG were stained for CD117 and CK5. A longitudinal view (a) and a cross section view (b) of the excretory duct are shown. Arrowheads indicate cells positive for both CD117 and CK5; arrows, cells positive for CD117 but not CK5; and double arrowheads, cells positive for CK5 but not CD117. (c) Dissociated cells prepared from the adult mouse SMG were stained for CD117 and CK5. Most of the CD117-expressing cells are negative for CK5; however, a small fraction of them are positive for CK5 (arrows). Nuclei are counterstained with DAPI. DIC, differential interference contrast microscopy. Scale bars = 25 μm (a, b), 50 μm (c).

Fig. 3. (a) Tissue sections of the adult SMG were stained for CD66a and AQP5. The intense CD66a staining is seen in AQP5-positive acini, extending to the AQP5-negative region (arrows) adjacent to the striated duct. (b–d) Dissociated cells were stained for CD66a and either AQP5, CLD4 or CK5. Most of the CD66a-intense cells are positive for AQP5, and most of the CD66a-weak cells are positive for CLD4 and CK5. Note some of the CD66a-intense cells are AQP5-negative and CLD4-positive. DIC, differential interference contrast microscopy. Scale bars = 25 μm.
Fig. 4. Tissue sections (a) and dissociated cells (b) of the adult mouse SMG were stained for CD117 and CD66a. Clusters of CD117-expressing cells (arrows) in the vicinity of the striated duct are intensely stained for CD66a. DIC, differential interference contrast microscopy. Scale bars = 25 μm.

0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 min at room temperature. After washing in PBS and incubation with 1% bovine serum albumin (BSA) in PBS for 10 min, specimens were incubated with primary antibodies diluted in 1% BSA/PBS for 1 h at room temperature or overnight at 4 °C, and then with fluorescein-conjugated secondary antibodies diluted in 1% BSA/PBS for 1 h at room temperature. After washing in PBS, specimens were mounted in ProLong Gold antifade reagent with DAPI (GIBCO) and observed with an Olympus BX-50 and Zeiss LSM700 microscope. Images were processed with ImageJ (NIH) and Photoshop (Adobe, San Jose, CA, USA).

Antibodies

Primary antibodies used were biotinylated mouse anti-CD66a (clone CC1) (BioLegend, San Diego, CA, USA), goat anti-CD117 (R&D, Minneapolis, MN, USA), rat anti-CD117 (clone ACK2) (BioLegend), goat anti-E-cadherin (R&D), mouse anti-E-cadherin (clone 36) (Transduction Laboratories, San Jose, CA, USA), rabbit anti-aquaporin 5 (Calbiochem, San Diego, CA, USA), rabbit anti-claudin 4 (provided by M. Furuse), rabbit anti-NKCC1 (provided by J. Turner), and rabbit anti-cytokeratin 5 (BioLegend). Secondary antibodies used were all donkey antibodies labeled with Alexa Fluor488 and
Results and discussion

Expression patterns of two cell surface molecules CD117 and CD66a were examined in the adult mouse SMG. Immunostaining of tissue sections showed that both molecules were expressed mainly in the epithelial tissue marked by E-cadherin (data not shown). Two different antibodies against CD117 gave similar staining patterns (data not shown). As the staining intensity for the two molecules varied within the tissue, their expression patterns were analyzed in detail with tissue sections and dissociated cells, comparing with the expression of several marker molecules.

CD117 expression in the SMG was compared with the acinar marker of the water channel aquaporin-5 (AQP5) (Delporte and Steinfeld, 2006) and the ductal marker of the tight junction protein claudin-4 (CLD4) (Hashizume et al., 2004; Peppi and Ghabriel, 2004). In tissue sections, CD117 expression was detectable in CLD4-positive ducts, but not in AQP5-positive acini (Fig. 1a and b), as previously reported (Lombaert et al., 2008, 2013; Nanduri et al., 2013). Close inspection in combination with DIC (differential interference contrast) observation demonstrated that CD117-expressing cells were distributed sparsely in the striated and excretory ducts and also clustered at the CLD4-positive region in the vicinity of the striated ducts (Fig. 1a). The CLD4 positivity, AQP5 negativity and the unique location of the clustering CD117-expressing cells suggested that they resided in the intercalated duct region, confirming the recent report by Lombaert et al. (2013). The number of CD117-expressing cells appeared larger in the excretory duct than the striated duct. Confirming the tissue section observations, analysis with dissociated cells prepared from the SMG epithelium-rich fraction showed that CD117 was detectable in some of the CLD4-positive cells (Fig. 1c) but not in the AQP5-positive cells (Fig. 1d).

CD117 expression was then compared with that of cytokeratin 5 (CK5), which is expressed by the basal cells of the excretory ducts and myoepithelial cells surrounding the acini and intercalated ducts. Although the expression of CD117 and CK5 are involved with stem/progenitor cells of salivary glands (Lombaert et al., 2008; Feng et al., 2009; Knox et al., 2010; Lombaert and Hoffman, 2010; Xiao et al., 2014), comparative analyses of their expression patterns in the adult glands have not been reported. In the excretory duct, four types of cells regarding the expression of CD117 and CK5 were recognized, including cells positive for both CD117 and CK5, positive either for CD117 or for CK5, and negative for both molecules (Fig. 2a and b). These cells were frequently noticed to associate with each other. CK5-positive cells also surrounded the CD117-expressing cell clustering in the intercalated duct region; however, co-localization of CD117 and CK5 was not recognizable (Fig. 2a). Together with the finding that the CD117-expressing cell clusters were positive for the tight junction protein CLD4 (Fig. 1a), it is suggested that these cells are the luminal cells of intercalated ducts, but not myoepithelial cells. CK5-positive cells were undetectable in the striated duct. Consistent with the tissue section observations, only a small fraction of CD117-expressing cells was positive for CK5 (Fig. 2c).

In contrast to CD117, CD66a was expressed in the entire epithelium, with the staining intensity different between acini and ducts. CD66a staining in tissue sections was intense in acini, overlapping with AQP5 staining at the luminal cell surfaces, and was faint in the striated and excretory ducts. Notably, the intense CD66a staining extended from the AQP5-positive acini to the AQP5-negative region adjacent to the striated ducts (Fig. 3a). Analysis with dissociated cells also showed the existence of two populations of CD66a-positive cells, that is, CD66a-intense and -weak populations. All of the AQP5-positive cells were CD66a-intense (Fig. 3b), most of the CLD4-positive cells were CD66a-weak while the rest of them were CD66a-intense (Fig. 3c), and all of the CK5-positive cells were CD66a-weak (Fig. 3d).

As expected from the above data, the CD117-positive, intercalated duct cells were intensely stained with CD66a (Fig. 4a). Analysis with dissociated cells also showed that some of the CD117-positive cells were intensely stained for CD66a (Fig. 4b). The above findings together indicate that several cell types consisting of the SMG epithelial tissue are distinguished by combinations of two cell surface molecules.
surface molecules CD117 and CD66a, including acinar cells that are CD117-negative and CD66a-intense, intercalated duct cells that are CD117-positive and CD66a-intense, some of the striated and excretory ducts that are CD117-positive and CD66a-weak, and the rest of the ductal cells and myoepithelial cells that are CD117-negative and CD66a-weak.

Like CD117-expressing cells, Ascl3-expressing progenitor cells of the SMG were previously shown to reside sparsely in the striated and excretory ducts, co-localizing with cells expressing NKCC1, a sodium–potassium–chloride co-transporter (He et al., 1997; Arany et al., 2011). As revealed by double staining of tissue sections, CD117 staining indeed overlapped with NKCC1 staining in the ductal parts (Fig. 5). As far as examined, cells of the ductal parts were either positive or negative for both CD117 and NKCC1. In addition, the CD117-expressing intercalated duct cells clustering near the striated ducts were positive for NKCC1. Thus, it is suggested that the Ascl3-expressing progenitor cells is a subpopulation of the CD117-expressing cells. As many CD117-expressing cells were negative for CK5 (Fig. 2), the CD117-positive, CK5-negative population may include the Ascl3-expressing progenitor cells.

Recently, it was demonstrated that, of the CD117-expressing cells isolated from the adult mouse SMG, Lin−CD24−CD117+Scal+ population exhibited the highest activity for proliferation, self-renewal and differentiation in vitro and in vivo (Xiao et al., 2014). The residential site of Lin−CD24−CD117+Scal+ stem cells in the SMG is not clear; however, the stem cell population was reported to include CK5-expressing cells and our data showed that a small fraction of the CD117-expressing cells resident in the excretory duct were positive for CK5. Thus, some of the Lin−CD24−CD117+Scal+ stem cells likely reside in the excretory duct. Our data confirm the notion that there exist multiple types of stem/progenitor cells in the CD117-expressing population of the adult mouse SMG (Lombaert et al., 2013; Nanduri et al., 2013).

In conclusion, our present study shows that several types of SMG-constituting cells are discriminated by differential expression of CD117 and CD66a, confirming the heterogeneous nature of the CD117-expressing progenitor cells resident in the ducts. The use of the two cell surface markers may be useful to isolate and analyze the molecular and cellular nature of the CD117-expressing progenitor cells.

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