Journal of Cellular Biochemistry 103:284–295 (2008)

Matrigel-Induced Acinar Differentiation is Followed by Apoptosis in HSG Cells

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Abstract It has been shown that a human salivary gland cell line (HSG) is capable of differentiation into gland-like structures, though little is known of how morphological features are formed or controlled. Here we investigated the changes in cell proliferation and apoptosis upon terminal differentiation of HSG cells in Matrigel, an extracellular matrix derivative. Changes in the expression of survivin, a prominent anti-apoptotic factor, and caspase-3, a key apoptotic factor were also measured. In order to better understand the involvement of key signal transduction pathways in this system we pharmacologically blocked the activity of tyrosine kinase, nuclear factor kappa B(NFκB), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and matrix metalloproteases (MMP). Results of these studies demonstrate that cytodifferentiation of HSG cells to an acinar phenotype is accompanied first by a decrease of cell proliferation and then by a massive programmed cell death, affected by multiple signal transduction pathways. Thus, Matrigel alone is insufficient for the full maturation and long term survival of the newly formed acini: the presence of other factors is necessary to complete the acinar differentiation of HSG cells. J. Cell. Biochem. 103: 284–295, 2008.

Key words: HSG; differentiation; apoptosis; extracellular matrix; acinar; survivin; caspase

Acinar cells of the salivary glands are responsible for the secretion of electrolytes, water, and most salivary proteins, whereas ductal cells largely modify the primary secretion. Acinar cells can be permanently destroyed by radiotherapy or Sjögren's syndrome leading to reduced salivary function and disastrous consequences for oral health and quality of life. Activation of pluripotent progenitor cells could be a potential source for renewal of ablated acini. There is increasing evidence to show that multipotent progenitor cells in salivary glands are localized to the intercalated ducts [Eversole, 1971; Vugman and Hand, 1995; Kishi et al., 2006]. These progenitor cells contribute to the renewal of acinar cells via differentiation, though acinar cells may also be renewed by autologous cell division [Denny and Denny, 1999].

The HSG cell line originates from human salivary gland intercalated duct [Shirasuna et al., 1981], and so is regarded as a potential model for the repopulation of damaged acinar cells from their progenitors. Notably, HSG cells are capable of in vitro morphodifferentiation to acinar cells by culture on basement membrane

Grant sponsor: Hungarian National Scientific Research Fund (OTKA); Grant numbers: 61543, 42558; Grant sponsor: Hungarian Ministry of Education; Grant number: OM 18657/2005; Grant sponsor: The Wellcome Trust (Travelling Research Fellowship Grant); Grant number: 071268/Z/03/Z, 2003.

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Received 10 April 2007; Accepted 11 April 2007

DOI 10.1002/jcb.21404

extracts or laminin and several investigators have described these early morphological and functional changes [Royce et al., 1993; Hoffman et al., 1996; Zheng et al., 1998; Jung et al., 2000; Crema et al., 2006; Vág et al., 2007].

Salivary gland morphogenesis requires a complex interaction between cell proliferation, apoptosis and histodifferentiation. Epithelial cell proliferation occurs at all stages of gland development, whereas apoptosis begins with the onset of lumen formation at an early phase of salivary tissue formation [Jaskoll and Melnick, 1999]. The regulation of apoptosis is critical for developmental homeostasis and normal morphogenesis of embryonic tissues. Furthermore, apoptosis may have an important role in the pathophysiology of the salivary gland, such as in the destruction of salivary acini in Sjögren's syndrome [Manganelli and Fietta, 2003]. Because of the simplicity of the system, it seems appropriate to investigate the changes in cell proliferation and apoptotic processes upon terminal differentiation of HSG cells on Matrigel. We also studied the change of expression of survivin (a prominent anti-apoptotic factor) and also caspase-3 (a key apoptotic factor) during the course of in vitro differentiation. Finally, to better understand the involvement of key signal transduction pathways in the development of cell proliferation and apoptotic changes, we blocked several key mediators: tyrosine kinase, nuclear factor kappa B (NFκB), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and matrix metalloproteases (MMP). Our data confirm that undifferentiated HSG cells go through acinar morphogenesis when cultured in Matrigel. In addition, our results clearly show that cytodifferentiation is accompanied first by a decrease of cell proliferation and then by an extensive programmed cell death in HSG cell culture.

MATERIALS AND METHODS

HSG Cell Culture and Differentiation in Matrigel

HSG cells from human submandibular origin [Shirasuna et al., 1981] were grown on plastic tissue culture dishes (BD Biosciences, Franklin Lakes, NJ) at 37°C in 5% CO_2 air. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, SIGMA-Aldrich, Saint Louis, MO), 10% sterile filtered fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). Culture medium was changed three times a week and the cells were passaged using 0.25% trypsin/1.0 mM EDTA (Sigma) before reaching confluence. Growth Factor Reduced Matrigel (BD Biosciences) was used for the differentiation of HSG cells at a concentration of 8.5 mg/ml. Matrigel was thawed on ice and added to HSG cell-suspensions (1:1 ratio) using pre-cooled pipettes. The plates coated with the HSG-Matrigel suspension were incubated at 37°C for 1 h. Cell numbers were adjusted to 2.5×10^4 cells/cm^2.

Cell Proliferation/Viability Assay and DNA Synthesis

Cell viability was assessed by the XTT assay kit (Sigma) according to the instructions of the manufacturer. Briefly, medium containing the cells was seeded into 96-well plates and cultured for 1–6 days. Reconstituted XTT was added to each well (20% volume of the medium) and the cells were incubated at 37°C for two hours. Absorbance of the samples was measured at 450 nm (Bio-Rad 3550 spectrophotometer, Bio-Rad Laboratories, Hercules, CA) with the reference wavelength of 690 nm.

The rate of DNA synthesis was estimated by ^{3}H-thymidine incorporation. Incorporation of thymidine into DNA was measured after cells were cultured either on Matrigel or on plastic for various time periods (20 h, 26 h, 2 days and 3 days) [Rácz et al., 2006]. Cell cultures were exposed to 1 μCi/ml [^{3}H] thymidine in serum free tissue culture medium for 2 h. Dispase (Roche Diagnostics GmbH, Roche Applied Science, Penzbeg, Germany, 2.4 U/ml, 1 h, room temperature) was used to isolate the HSG cells from Matrigel and plastic after removing the radiolabeled culture medium. Cell suspensions were centrifuged (800*g*; 5 min) and the supernatant was discarded. After double PBS washes (800*g*, 5 min) the radiaoctive pellet was precipitated using 10% trichloracetic acid and washed twice with PBS. The precipitate was dissolved in 400 μl 1 M NaOH and 100 μl aliquots were mixed with 3 ml of scintillation fluid (Opti Phase HiSafe II, PerkinElmer, Wellesley, MA). Radioactivity was determined by liquid scintillation spectrophotometry (Wallac 1409, PerkinElmer).

Flow Cytometry

HSG cells grown on plastic or Matrigel were isolated and dissociated to single cell suspensions with dispase (1 h) and 0.02% EDTA in

phosphate buffer saline (PBS, 20 min). Cells were washed in PBS and centrifuged (800*g* for 5 min). HSG cells were resuspended in Annexin-Binding Buffer (Central European Biosystem, Brno, Czech Republic, 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and 5 μl annexin-V-FITC (Central European Biosystem) was added to 100 μl aliquots of 1×10^6 cell/ml cell suspensions. The cell-annexin-V-FITC mixture was incubated for 15 min at room temperature in the dark. After incubation, the mixture was diluted by adding 400 μl of Annexin Binding Buffer. Mixed cell suspensions were analyzed by FACS Vantage (BD Biosciences). The FITC was excited at 488 nm and fluorescence was detected using a band-pass filter of 530/15 (FL1). The percentage of annexin-V-FITC positive cells was determined in 5,000 cells. Two fluorescent cell populations were gated in each sample. LA represents low level of annexin binding, while HA represents a high level of annexin binding cell surface structures.

Real Time PCR

Total RNA from HSG cells was isolated by TRI Reagent (Sigma) and RNA was cleaned up using an RNeasy micro kit (Qiagen Ltd, Crawley, UK) with on-column DNase digestion. The concentration of the RNA was determined by the Ribogreen method (Invitrogen, Carlsbad, CA). The integrity of the RNA was verified by electrophoresis on a 1.2% agarose/formaldehyde gel and 200 ng total RNA was used, per sample, for cDNA synthesis using oligo-dT priming (TaqMan reverse transcriptase reagents, Applied Biosystems, Foster City, CA) in a total volume of 10 μl. Commercially available total RNA from human submandibular glands (BD Biosciences) as a biological control was also included in the cDNA synthesis.

For PCR amplification, 5% of the cDNA synthesis reaction was used with the real time PCR primers and target-specific fluorescence probe (FAM-labeled MGB probe). The probes and primers were selected from the Applied Biosystem Assay on Demands database for survivin (BIRC5) and for the human acidic ribosomal phosphoprotein PO (RPLPO), which was used as an internal control. Universal Mastermix (Applied Biosystems) containing AMPerase was used for amplification in 25 μl total PCR reaction. For detection of fluorescence signal during the PCR cycles, an ABI Prism Sequence Detection System 7700 (Applied Biosystems) was used with the default setting (50°C for 2 min, 95°C for 10 min, 45 cycle: 95°C for 15 s, 60°C for 1 min). Each treatment was repeated two to five times and each sample was measured in duplicate. The values of the threshold cycles were used for statistical comparison. Changes in gene expression levels were estimated by calculating the relative expression values normalized to the RPLPO level from the same sample.

Immunohistochemistry

HSG cells were plated on the surface of 12 mm-diameter microscope coverslips (Paul Marienfeld Gmbh, Lauda-Königshofen, Germany) with or without Matrigel. Cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and were permeablized with Triton X-100 (5 min, 0.1%v/v in PBS). Nonspecific antibody binding was blocked by incubation with 5% FCS in PBS at room temperature (1 h). The antibody for survivin (10 μg/ml, polyclonal rabbit, Sigma) was diluted with this solution and incubated overnight at room temperature. Second-layer antibody to anti-rabbit IgG conjugated with Alexa 488 (Invitrogen) was added to the samples at a dilution of 1:500 and incubated for 90 min at room temperature. Nuclei were stained with propidium-iodide (Sigma, 0.025 mg/ml) in 0.01% Triton X-100 for 30 min at room temperature. The stained preparations were evaluated using confocal laser scanning microscope (Olympus BX61-FV300, Olympus America, Center Valley, PA).

Caspase-3 Activity Measurement

Caspase-3 activity was estimated by using the caspase-3 fluorimetric assay kit (Sigma). The HSG cells were isolated from Matrigel and dissociated to single cells using dispase and 0.02% EDTA. HSG cells grown on plastic were detached using 0.02% EDTA. Isolated HSG cells were pelleted and washed with PBS by centrifugation at 600*g* for 5 min at 4°C. As per the kit protocol, the cell pellet was resuspended in 1× lysis buffer at a concentration of 5×10^6 cells per 500 μl and incubated on ice for 30 min. The samples were centrifuged at 13,000*g* for 15 min at 4°C. Forty microliters aliquots of supernatant were added to 2 ml reaction mix including 5 μl assay buffer. The fluorescence was measured at 360 nm excitation and 460 nm emission (Hitachi F-4500 Fluorescence Spectrophotometer, Tokyo, Japan). Values are given as percent of control.

Signaling Pathway Inhibitors

The selective MMP-2/MMP-9 inhibitor (2R)-2[(4-biphenylylsulfonyl)amino]-3-phenylpropionic acid (MMPi), the PI3K inhibitor LY 294002, the NFκB activation inhibitor 6-amino-4-(4-phenoxyphenylethylamino)-quinazoline (NFκBi) and the specific epidermal growth factor receptor tyrosine kinase inhibitor AG1478 were purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). The PKC inhibitor, staurosporine, was purchased from SIGMA. Cell viability was assessed by the XTT assay kit (Sigma) after application of different pathway inhibitors in the following concentrations: 10 μM MMPi, 10 μM LY 294002, 20 nM NFκBi, 250 nM AG1478, 100 nM staurosporine. Inhibitors were diluted into final concentration in the cell culture medium and applied for the 3 or 5 days.

Statistics

Data are given as mean ± SEM. Differences between experimental groups were tested by ANOVA followed by Bonferroni and Dunnett post-hoc tests.

RESULTS

Morphological changes of HSG cells cultured in Matrigel

As expected, HSG cells grown on a plastic surface formed monolayers, first making epithelial fields and then proliferating continuously until confluency (Fig. 1A). In growth factor reduced Matrigel (4.55 mg/ml final concentration), HSG cells formed spherical structures within 24 h and developed microvilli on the cell surfaces (Fig. 1B). Increasing numbers of cells formed acinar structures by the next day (Fig. 1C). Dense granules appeared in the bodies of the acinar-like cells at Day 4 and lumen formation was detectable in the center of the acinar structures (Fig. 1E,F), which also exhibited strong amylase expression (Fig. 1G). Most 4 days acini in Matrigel included 10–12 cells. After Day 5 the acinar structures disintegrated, most of the cells lost contact with each other and were shrunken in appearance (Fig. 1D). Confocal micrographs from the equatorial plan of propidium iodide stained nuclei showed dense and homogenous chromatin in plastic-cultured HSG cells (Fig. 1J), while nuclei were fragmented in Matrigel cultures on Day 5 (Fig. 1K).

In consecutive experiments HSG cells were cultured for three days in Matrigel, then isolated carefully from the Matrigel with dispase (so as not to destroy the acini) and then remixed with fresh Matrigel. The integrity and growth of replated acini were followed by phase-contrast microscopy. These replated acini disintegrated after 2–3 days in the fresh Matrigel in a very similar manner to the acini grown in Matrigel for 5–6 days without replating (Fig. 1I). In other experiments, HSG cells cultured in Matrigel for 3 or 6 days were recovered, and single cell

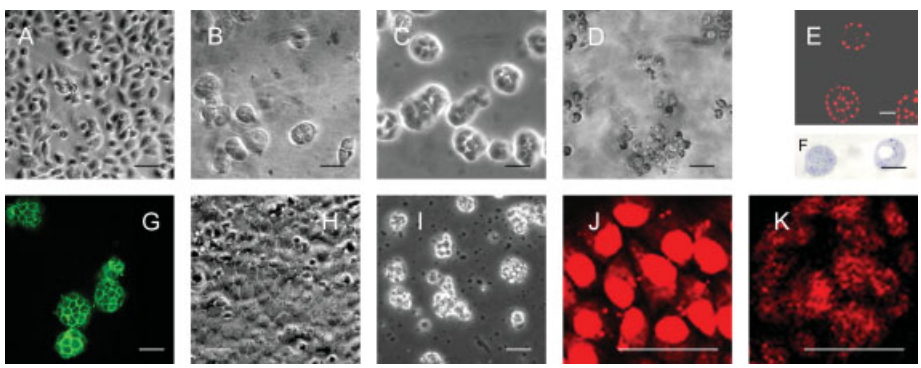

Fig. 1. Acinar differentiation of HSG cells in Matrigel. Undifferentiated HSG cells on a plastic surface grew as an epithelial monolayer with cobblestone appearance (**A**). HSG cells cultured in Matrigel formed acinar structure in 2 days (**B**) and still maintained this structure after 4 days of incubation (**C**). On Day 6 most of the acini disintegrated and cells had a shrunken appearance (**D**). Propidium iodide nuclear staining visualized by confocal microscopy revealed that an acinus consists of 20–50 cells and the outer cells appear polarized (**E**). Hematoxylin-eosin staining shows discrete spheroid acini (**F**). Intense α-amylase staining in HSG cells cultured in Matrigel was demonstrated by immunofluorescence staining observed with confocal microscopy (**G**). HSG cells isolated from Matrigel after 3 days and replated onto a plastic surface formed monolayers and proliferated (**H**). Acini disintegrated after 2–3 days in fresh Matrigel, similar to acini grown in Matrigel for 5–6 days without replating (**I**). Photos A–D, H, and I were taken by phase-contrast microscope. Confocal micrographs from the equatorial plan of propidium iodide stained nuclei showed dense and homogenous chromatin in plastic-cultured HSG cells (**J**), while nuclei were fragmented in Matrigel cultures on Day 5 (**K**).

suspensions were replated onto plastic culture plates. HSG cells originating from 3-day Matrigel acini attached to the plastic surface, while HSG cells originating from 6-day Matrigel acini failed to attach. Cells originating from 3 days in Matrigel, replated on plastic, formed again undifferentiated, cobblestone patches, suggesting the reversibility of the differentiation after 3 days of Matrigel culture (Fig. 1H).

Effect of Matrigel on Cell Proliferation, Viability and DNA Synthesis of HSG Cells

Cell proliferation and viability of HSG cultures grown either on plastic surface or embedded in Matrigel were evaluated first by XTT assay (Fig. 2A). When cells were grown on plastic, a growth curve was observed that is characteristic of immortal cells: a rapid increase in cell number until confluency was reached, followed by a plateau and/or a modest decline. This decline, observed at Day 6, is primarily due to the loss of firm contact of cells with the plastic surface; these cells are subsequently removed when the culture dish is rinsed. HSG cell numbers in Matrigel exhibited a very different curve during the 6-day course of the experiment. During the first 3 days in culture, cell growth was similar—though slightly lower—on Matrigel compared to plastic, suggesting that cell proliferation is still considerable during this period. At Day 4, however, HSG cell proliferation in Matrigel slowed noticeably, followed by a dramatic decline of cell numbers at Days 5 and 6. Together with the morphological changes described above, our data suggest that in Matrigel HSG cells first proliferate and differentiate, but soon after that extensive cell death occurs in the culture.

In line with the cell viability data, DNA synthesis did not differ during the first cell cycle (i.e., within 20 h) between cells grown on plastic or Matrigel (Fig. 2B). Thymidine incorporation significantly decreased at 26 h, 2 and 4 days, in Matrigel cultures compared to cells grown on plastic, suggesting an inhibition of proliferation during this period (Fig. 2B).

In a separate set of experiments, HSG cells cultured in Matrigel for 3 or 6 days were recovered by enzymatic digestion of Matrigel. The recovered single cell suspensions were replated onto plastic culture plates. Following a further 2 days of cultivation on plastic, ^{3}H-thymidine incorporation into the replated cells was measured and compared to those of 2-day HSG cells cultured only on plastic (control group). The level of DNA synthesis in the 3-day Matrigel replated cells ($98.4 \pm 4.3\%$ of the control group) did not differ from the control cells grown on plastic. HSG cells replated from 6-day-Matrigel acini failed to survive, and the DNA synthesis of these cells was practically undetectable.

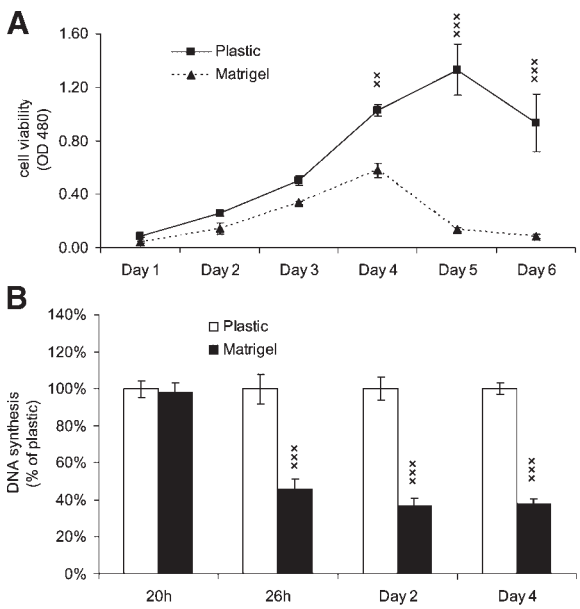

Fig. 2. Cell viability and DNA synthesis of HSG cells cultured in Matrigel or on a plastic surface. The effect of incubation time on HSG cell viability assessed by means of the XTT assay (n = 5–5) (**A**) and on DNA synthesis measured by ^{3}H-thymidine incorporation (n = 8–8) (**B**). Results are reported as the mean ± SE. The effect of incubation time ($P < 0.001$), the effect of the surface ($P < 0.001$) and their interaction ($P < 0.001$) were significant by two-way ANOVA both in the XTT assay and ^{3}H-thymidine incorporation. *** indicates the significance ($P < 0.001$) of the pair-wised comparison of Matrigel versus plastic tested by Bonferroni test.

Apoptosis Detection by Annexin Binding

Cells grown on plastic showed a very low level of annexin binding, even after 5 or 6 days in culture. In contrast, when grown in Matrigel, the percentage of annexin-V-FITC positive cells in the investigated HSG cell population increased with the length of incubation. The proportion of cells with a high level of annexin binding (HA fraction) gradually increased over the fraction having low annexin binding (LA fraction), suggesting not only a qualitative but also a quantitative relationship between annexin binding and the progress of apoptosis (Fig. 3).

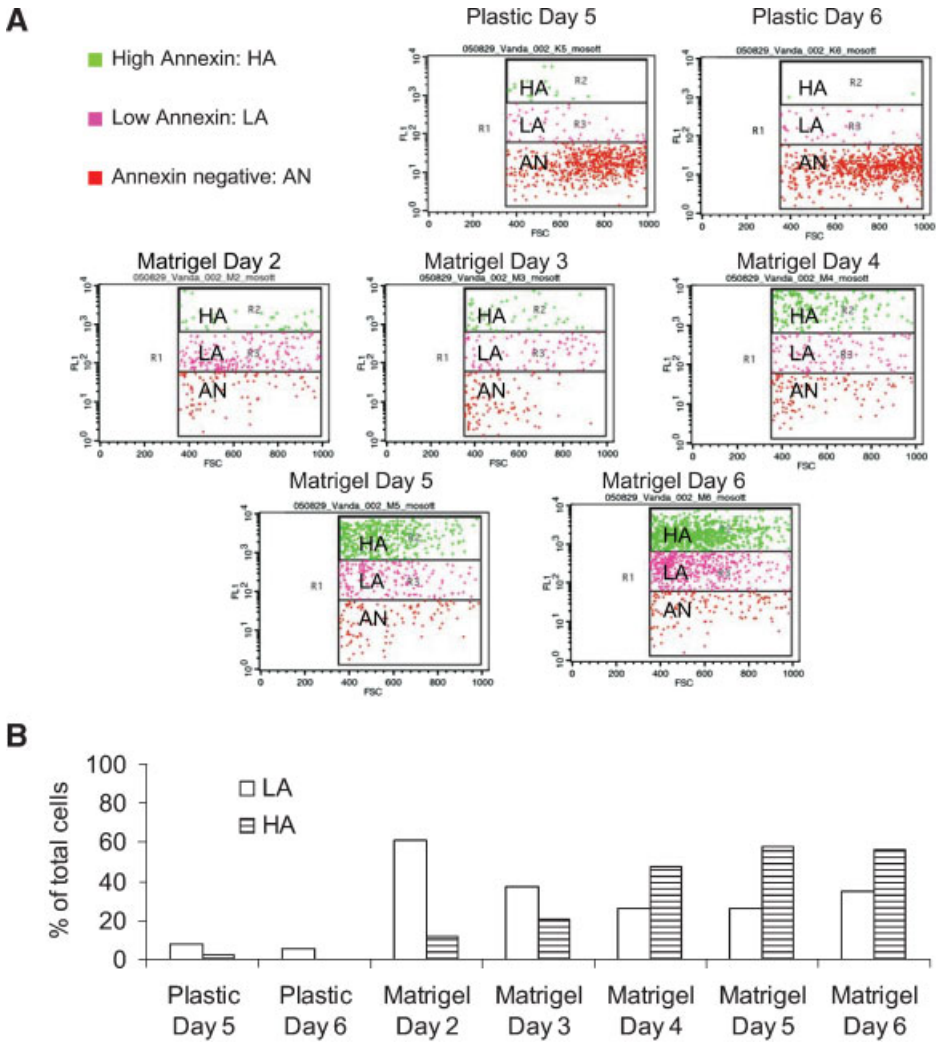

Fig. 3. Apoptotic progression of HSG cells cultured in Matrigel. The percentage of annexin -V-FITC positive cells was determined by FACS, from 5,000 cell size events (**A**). Cells grown on plastic showed very low levels of annexin binding, even after 5 or 6 days in culture. In Matrigel, the percentage of annexin V-FITC positive cells increased with the time of incubation. Two fluorescent cell populations were gated in each sample according to the fluorescence intensity: Low annexin (LA) where $10^2 < FL1 < 10^3$ and High annexin (HA) where $10^3 < FL1 < 10^4$. The annexin negative population is marked by AN. The proportion of cells in Matrigel with a high level of annexin binding (HA fraction) gradually increased over the fraction representing low level annexin binding (LA) (**B**) during the 6 days of incubation.

Survivin Expression and Caspase-3 Activity of HSG Cells in Matrigel

Survivin mRNA expression in the HSG cultures, evaluated by quantitative PCR, was similar for the first 3 days of incubation in Matrigel and on plastic. At Day 5, survivin mRNA expression decreased markedly in Matrigel compared to the cells on plastic (Fig. 4F). As the last column shows, survivin mRNA expression was much lower in normal human submandibular tissue than in HSG cells either on plastic or in Matrigel (Fig. 4F).

Both the nuclei and the cytoplasm of undifferentated HSG cells grown on the plastic surface showed survivin immunopositivity. Therefore, in these cells the nucleus appears to be yellow, exhibiting the overlap of the red propidium iodide dye and the green survivin staining (Fig. 4A). Survivin could still be detected in the cytoplasm, but not in the nuclei, of acinar-like HSG cells at Day 4, cultured in Matrigel (Fig. 4B,C). The cytoplasm of the few compact acini at Day 6 was still survivin immunoreactive, whereas the survivin protein could not be detected in the disintegrating acini (Fig. 4D,E).

Caspase-3 activity changed in an inverse manner compared to survivin expression. When compared to the activity of cells grown on plastic

(100 ± 49%), caspase-3 activity did not change in culture at Days 1 and 2 (79 ± 5 and 176 ± 72%, respectively), but a significant increase was observed at Day 3 in Matrigel (446 ± 11%, $P < 0.001$, n = 3–3), suggesting the activation of caspase three-dependent apoptotic processes.

Effect of Major Signaling Pathway Inhibitors on cell Viability and Morphology of HSG Cells

This part of the study was performed to gain some insight as to the involvement of major intracellular signaling pathways involved in the Matrigel-induced proliferative and apoptotic processes. The application of various inhibitors caused similar changes, regardless of the number of days (3 vs. 5) of incubation (Fig. 5). PKC and NFκB inhibitors decreased the proliferation of cells both on plastic and in Matrigel, compared to the cultures where no inhibitors were used (Fig. 5). The inhibitory effect of NFκB on growth rate was much more pronounced in Matrigel (interaction: $P < 0.001$). In the presence of PI3K inhibitor the proliferation significantly decreased on plastic, but was unchanged in Matrigel. Contrary to these observations, when MMP inhibitor was applied the proliferation was not affected on plastic, but was slightly increased in Matrigel. Finally, tyrosine kinase inhibition slightly decreased the number of the cells on plastic, but significantly increased numbers on Matrigel, thus attenuating the Matrigel-induced effects (Fig. 5).

When observing morphological changes in Matrigel cultures in response to the inhibitors at Day 3, the most pronounced change was the appearance of small, shrunken, undifferentiated cells with stretched cytoplasm when staurosporine or the NFκB inhibitor was used. On Day 5, all of the Matrigel cultures disintegrated regardless of whether any inhibitor or none was applied.

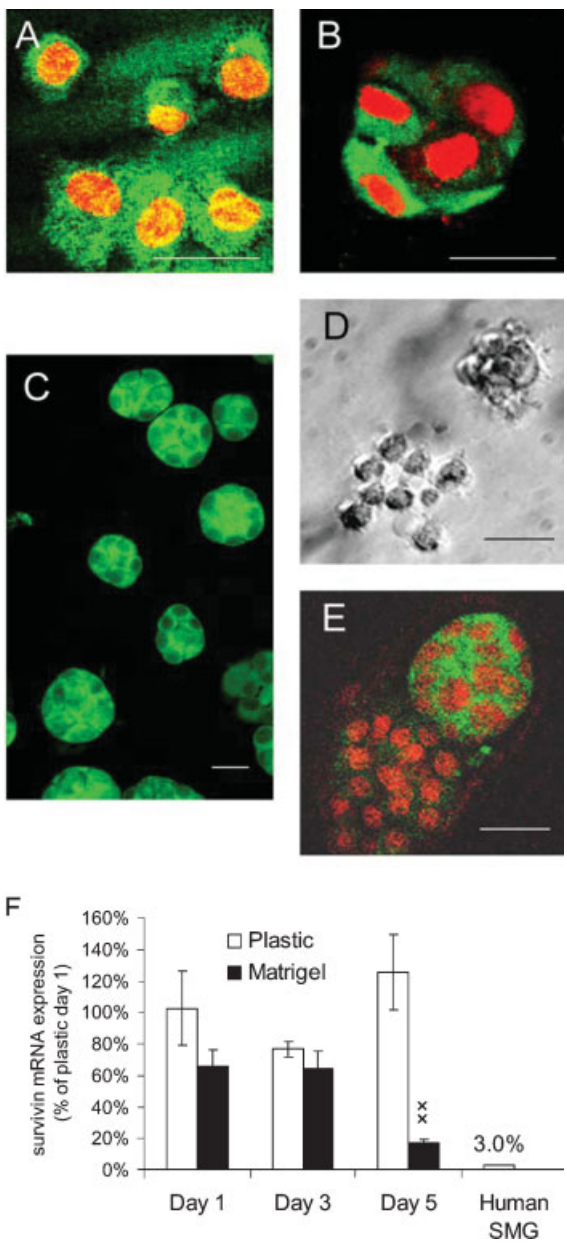

Fig. 4. Survivin protein expression in HSG cells cultured in Matrigel, or on a plastic surface. Immunofluorescence staining of survivin (Alexa 488) with propidium iodide nuclear staining revealed protein expression both in the cytoplasm and nucleus of HSG cells on plastic (**A**): survivin was expressed only in the cytoplasm of HSG cells cultured in Matrigel for 4 days (**B**). Immunofluorescence staining of survivin without propidium iodide nuclear staining confirmed the lack of survivin in the nucleus of HSG cells in Matrigel (**C**). After 6 days of incubation in Matrigel the phase-contrast examination showed compact acini (**upper right** corner in **D**) as well as disintegrated acini (**lower left** corner in D). Immunofluorescence staining shows the disappearance of survivin from the disintegrated acinus, but survivin is still highly expressed in the cytoplasm of the intact acinus (**E**). Quantitatively, survivin mRNA expression in HSG cells cultured in Matrigel for 1 or 3 days was similar to that in cells on plastic, but at Day 5 the survivin expression was significantly higher in cells in Matrigel (n = 3–6) (**F**). Total RNA prepared from 24 normal human submandibular gland (SMG, Clontech Premium RNA) was used as a reference. Data are reported as mean ± SE. The effect of the surface ($P < 0.001$), but not the effect of incubation time (NS) was significant by 2-way ANOVA. ** indicates the significance ($P < 0.01$) of the pair-wised comparison of Matrigel versus plastic tested by Bonferroni test.

DISCUSSION

Irreversible loss of functional salivary tissue is common clinically, devastating for oral health and quality of life and difficult to treat. The idea

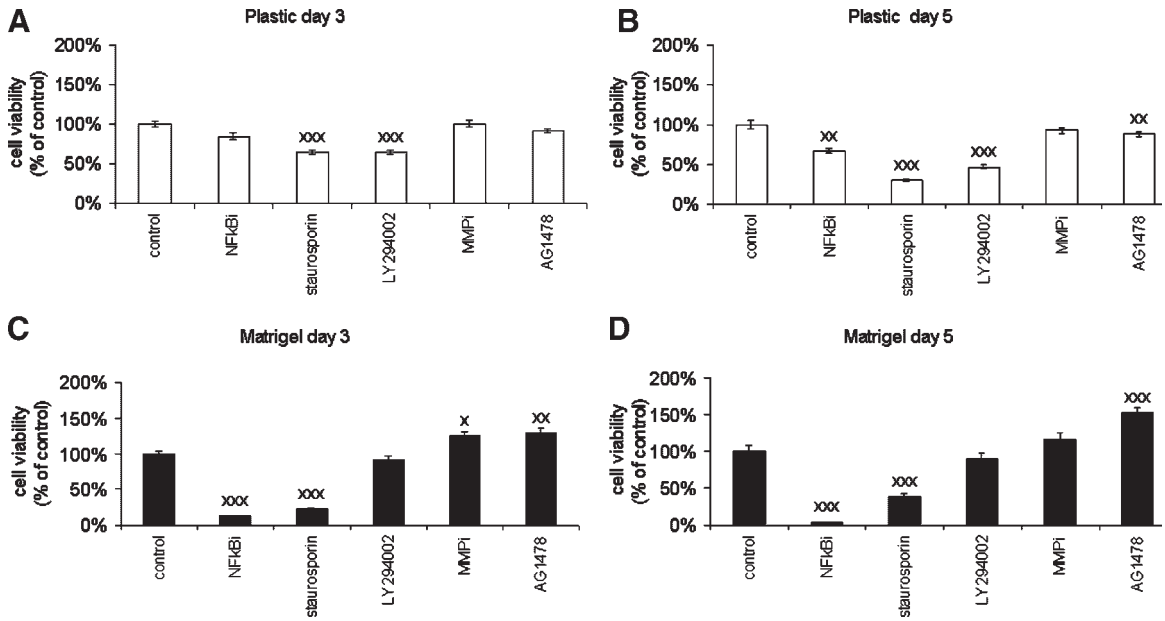

Fig. 5. Effects of various signal transduction pathway inhibitors on cell viability of HSG cells cultured in Matrigel or on a plastic surface for either 3 or 5 days. Cell viability was assessed by the XTT assay following the application of the pathway inhibitors for the full culture period in the following concentrations: 20 nM NFκBi, 100 nM staurosporine (for PKC inhibition), 10 μM LY 294002 (for PI3K inhibition), 10 μM MMPi, and 250 nM AG1478 (for tyrosine kinase inhibition). Data are reported as mean ± SE in% of the untreated control (n = 8–8). Effects of inhibitors were tested by comparing the treated cells to the untreated controls of the same experimental day, cultured either in Matrigel or on plastic surface. Effect of inhibitors was significant ($P < 0.001$) as it was analyzed by 1-way ANOVA separately for each culture conditions (**A**–**D**). Dunnett test was performed to compare treated samples to the control ones: $^{\times}P < 0.05$, $^{\times\times}P < 0.01$, $^{\times\times\times}P < 0.001$.

of repopulating a damaged gland with new cells is attractive and HSG cells have been proposed as a model of such cells, given their ability to form rudimentary acinar structures when cultured on extracellular matrix derivatives. Even for 'proof of principle' it would be necessary to follow the fate of differentiated HSG cells and encourage their organization into higher order glandular structures. In the present study HSG cells formed spheroid structures embedded in Matrigel, in agreement with findings of others [Royce et al., 1993; Hoffman et al., 1996; Crema et al., 2006; Vág et al., 2007]. However, instead of further development the differentiated HSG cells proceeded to lose their immortality and underwent apoptosis. This process was accompanied by the gradual disappearance of the anti-apoptotic protein, survivin, from the nucleus and cytoplasm and an increase in caspase-3 activity. HSG cells, which otherwise exhibit immortal characteristics, have previously been shown to undergo programmed cell death in response to strong cytotoxic conditions such as TNFα exposure [Kulkarni et al., 2006], extremely high intracellular Ca^{2+} levels [Atsumi et al., 2006a] and induction of reactive oxygen species [Atsumi et al., 2006b], but not in response to an extracellular matrix derivative, which is generally regarded as a differentiating agent. Vág et al. [2007] also observed multiply cell death in HSG culture 5 days after plating the cells on basal membrane extract.

This study suggests that the pro-apoptotic changes in HSG cells developed gradually, but there is a point of no return between Days 3 and 4 when survivin expression is markedly decreased, while caspase-3 activity and annexin-V binding strongly increased. After 3 days of culture in Matrigel, HSG cell growth arrest and apoptosis were reversible by replating the cells on plastic dishes; after 4 days on Matrigel the cells failed to survive, even after replating. A possible explanation for the short survival period of differentiated HSG cells is aging of the basement membrane. Apoptosis has been reported in various epithelial cells due to loss of contact with the extracellular matrix [Frisch and Francis, 1994; Boudreau et al., 1995; Dirami et al., 1995]. In our experiments, apoptosis was apparently not evoked by the matrix degradation-induced anoikis (i.e., the loss of contact with extracellular matrix) since HSG

cells, kept in Matrigel for 3 days then replated into new Matrigel for another 3 days, went through the same apoptotic process as cells cultured for 6 days in the same Matrigel. These data also suggest that Matrigel alone is an inadequate environment for the longer survival and development of mature acini; the presence of other factors is required to achieve complete acinar differentiation of HSG cells.

It was notable that Matrigel inhibited DNA synthesis from the second day in culture, which is consistent with the findings of similar studies. A 50% reduction was found by Royce in ^{3}H-thymidine incorporation after 3 days in cells cultured on Matrigel compared to cells cultured on plastic [Royce et al., 1993]. Another study showed that the number of HSG cells measured by viability assays was somewhat lower on Matrigel compared plastic [Hoffman et al., 1998] during the course of the first 3 days. Lam et al. [2005] counted HSG cells on days 1, 2, 3, and 5, and found little difference between cell numbers cultured on plastic or Matrigel. This observation is consistent with our result because the peak cell population on Matrigel occurred at Day 4 followed by a decline on Day 5.

The loss of plasma membrane asymmetry is an early event in apoptosis resulting in the exposure of phosphatydylserine (PS) residues at the outer plasma membrane leaflet [van Engeland et al., 1997]. Since annexin strongly and specifically binds to PS, it can be used to detect apoptosis. In parallel with HSG cell differentiation we found the percentage of annexin-V-FITC positive cells gradually increased from Day 2, and the percentage of cells with high fluorescence (HA fraction) increased dramatically from the Day 3. Caspase-3 activity increased only at Day 4, contrary to the appearance of annexin positive cells after 2 days, suggesting that PS exposure may be mediated in part by a caspase-3-independent pathway as well. It is generally assumed that PS exposure is a consequence of caspase activation, though caspase-independent PS externalization has been reported in normal [Brown et al., 2000; Ferraro-Peyret et al., 2002] and transformed cells [Huigsloot et al., 2001]. PS exposure can occur with non-lethal cell injury such as stress, prior to the irreversible morphological changes [Martin et al., 2000]. The first 3 days culture in Matrigel may represent apoptotic stress for HSG cells, which can be reversed upon removal of the Matrigel. After 3 days, however, the cells irreversibly become committed to programmed cell death.

Survivin is expressed during fetal development, down-regulated in most terminally differentiated cells [Adida et al., 1998; Altieri, 2001], but elevated in numerous human cancer tissues that are associated with a poor prognosis [Adida et al., 2000a,b; Altieri, 2001; Ikehara et al., 2002; Ku et al., 2004]. It is known as an anti-apoptotic factor, negatively interfering with apoptosis, directly inhibiting caspase-3 and caspase-7 activities and regulating the G2/M phase of the cell cycle by associating with mitotic spindle microtubules [Ambrosini et al., 1997]. Indeed, survivin gene expression was much higher in undifferentiated HSG cells than in normal human salivary tissue, suggesting that it is one of the regulators of HSG cell immortality. The level of survivin mRNA considerable decreased during the course of apopotosis resulting in the rapid death of HSG cells. Consistent with this observation, nuclei and cytoplasm of undifferentated HSG cells grown on plastic were found to be survivin immunoreactive: on Matrigel the immunoreactivity was lost first in the nuclei then in the cytoplasm.

NFκB is an important transcription factor which plays a critical role in apoptotic processes. Activation of NFκB was shown to delay and protect against apoptosis [Yan et al., 2005; Toruner et al., 2006]. Based on this fact, inhibition of NFκB-mediated gene transcription is used to sensitize tumor cells for programmed cell death in cancer therapy [Saitou et al., 2005; Song et al., 2006]. The inhibition of NFκB nuclear translocation in submandibular gland explants from embryonic mice results in a significant increase in apoptosis and decrease in cell proliferation [Melnick et al., 2001]. NFκB was shown to be constitutively active in salivary gland cell lines and blocking of NFκB with an antisense oligonucleotide reduced the growth rate of HSG cells [Azuma et al., 1999]. Likewise, in the present study the pharmacological inhibition of NFκB [Tobe et al., 2003] caused a slight reduction in the cell number of HSG cells cultured on plastic that became much more pronounced in Matrigel. At the same time, HSG cells on Matrigel did not differentiate into acini in the presence of NFκB inhibition. These observations suggest that NFκB activity is low in HSG cells grown on plastic, but it becomes highly active in Matrigel and participates in the redirection of HSG cells first into an acinar

phenotype, then towards apoptosis. Application of staurosporine, a non-selective PKC inhibitor [Verspohl and Wienecke, 1998], resulted in small, shrunken, undifferentiated HSG cells with stretched cytoplasms and decreased cell numbers on both in Matrigel and plastic. Staurosporine been shown to have a range of effects on cell function. At least five different PKC isoenzymes are expressed in HSG cells: PKC α, γ, δ, μ, and ζ were shown to be present by immunoblot analysis [Lam et al., 2001]. Among the PKC isoforms, α and δ have been proposed to play key roles in cell differentiation and proliferation [Jung et al., 2000]. Our results indicate a role for PKC in cell differentiation and survival, however, staurosporine is believed to trigger apoptotic cell death in several epithelial cells [Wood and Osborne, 1997; Charlot et al., 2006] and may have directly induced apoptosis in this study.

PI3K is a key protein downstream of activation of growth factor receptor or focal adhesions. PI3K activity is required for growth factor dependent survival and the activated form of PI3K is able to protect cells against death induced by detachment from extracellular matrix, that is, anoikis [Frisch, 2000]. In the present study, application of a PI3K inhibitor [Lipskaia et al., 2003] to the HSG cells cultured on plastic decreased cell proliferation, suggesting that the immortal characteristic of the HSG cells depends in part on PI3K activity. However, neither cell survival nor the morphology of cells was influenced by PI3K inhibition during Matrigel-induced differentiation and apoptosis.

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) have been suggested as regulators of apoptosis [Frisch et al., 1996]. TIMP-1 inhibits apoptosis by inducing the TIMP-1 specific cell survival pathways involving activation of focal adhesion kinase in several epithelial cells [Liu et al., 2005; Taube et al., 2006]. HSG cells have been shown to produce MMP2 and MMP9 [Wu et al., 1997], two possible targets for inhibition of MMP activity. In the present study, the cell number was slightly but significantly elevated in cells cultured in Matrigel at Day 3, but not at Day 5, when an MMP2/MMP9 inhibitor was applied [Roeb et al., 2005]. These data suggest that although MMPs may have a minor role, but are not the major mediators triggering programmed cell death induced by Matrigel in HSG cells.

EGF and other growth promoting factors have been shown to stimulate the proliferative activity of HSG cells [Ota et al., 1996; Sato et al., 1996]. The proliferative response to these factors is mediated by a phosphorylation cascade that starts with receptor-associated tyrosine kinase activation. In our experiments, inhibition of the tyrosine kinase activity [Fan et al., 1995] moderately decreased the proliferation of HSG cells on plastic, indicating that either autocrine growth factors and/or EGF in the serum-supplemented culture medium contribute the proliferation of undifferentiated HSG cells. Unexpectedly, inhibition of the tyrosine kinase activity during culture in Matrigel resulted in an increase in cell numbers implying that the activity of this enzyme is actually driving pro-apoptotic events under these conditions. This might be due to the potentially unpredictable results, observed by others as well, since activation of the tyrosine kinase mediated growth factor pathways may induce very different physiological responses depending on the magnitude of activation and also on the actual condition of the exposed cells [Memon et al., 2006a,b; Oliveira et al., 2006].

In conclusion, results of the present studies demonstrate that cytodifferentiation of HSG cells to an acinar phenotype is accompanied first by a decrease in cell proliferation and then by significant programmed cell death, which likely involves multiple signal transduction pathways. The data indicate that Matrigel alone is insufficient for the full maturation and long term survival of acini and other factors are required to achieve complete acinar differentiation of HSG cells.

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