Neutrophils Increase Oral Squamous Cell Carcinoma Invasion through an Invadopodia-Dependent Pathway

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Abstract

Neutrophils have recently been shown to promote invasion and correlate with a poor prognosis in different cancers, including head and neck squamous cell carcinomas. In this study, we analyze the effects of neutrophils in the invasion of oral squamous cell carcinoma (OSCC) using a combination of conditioned media, direct and indirect coculture of human peripheral blood neutrophils, and UMSCC47 cells (OSCC cell line). Invasion and matrix degradation were determined using a modified in vitro invasion assay and an invadopodia assay, respectively. UMSCC47 and neutrophil cocultures or conditioned media from cocultures increased UMSCC47 invasion, invadopodia formation, and matrix degradation. Further analysis revealed an increase in TNFα and IL8 in supernatants of cocultures compared with neutrophil or UMSCC47 cultures alone and that inhibition of TNFα and IL8 significantly decreased OSCC invasion. Our results show that neutrophils increase the invasiveness of OSCC through the activation of invadopodia and matrix degradation, suggesting a paracrine activation loop between the two cells. Importantly, the presence of neutrophils in the oral environment may modulate the clinical behavior of OSCC. Cancer Immunol Res; 3(11); 1218-26. ©2015 AACR.

Introduction

The overall poor clinical outcome of oral squamous cell carcinoma (OSCC) is associated with its late detection, local aggressiveness, and presence of metastatic disease. The precise mechanisms underlying metastatic spread, however, are not well understood. Neutrophils have an important role in cancer biology (1–3), including tumor progression (4, 5), metastasis (6), and extracellular traps (NET)-dependent tumor metastasis (7). Although the role of inflammation and the immune system in the progression of cancers has been studied extensively, only recently has it been investigated in the setting of OSCC (8–13). Trelakis and colleagues (11) have shown that neutrophil infiltration of head and neck squamous cell carcinoma (HNSCC) was correlated with poor clinical outcome, while Wang and colleagues (14) used tongue squamous cell carcinomas to show that neutrophil infiltration was associated with lymph node metastasis, higher clinical stage, and increased chances of tumor recurrence. These findings raised the possibility of a cross-talk and possible costimulation of cancer cells and neutrophils. In support of this hypothesis, Dumitru and colleagues have shown that neutrophils challenged with pharyngeal squamous cell carcinoma cells (FaDu) showed a strong activation of p38/MAPK, CREB, and p27, leading to an increase in chemotaxis, cell survival, and secretion of CCL4 and CXCL8. They also observed an increase in expression of MMP9 and CCL4 by CD66b-positive cells in human tumor sections (15).

Here, we investigate the effects of neutrophils on the invasiveness of OSCCs using a combination of OSCC cell lines (UMSCC1 and UMSCC47) and peripheral blood neutrophils. We provide evidence that neutrophils increase the invasiveness of OSCC, independent of direct contact between the two cells. Coculture with neutrophils increases invadopodia formation and matrix degradation in cancer cells. A significant increase in invasion, invadopodia formation, and degradation was also seen in cancer cells exposed to conditioned media from neutrophils and UMSCC47 cocultures. Further analyses suggest that the neutrophil-dependent increase in invasion observed is based on a paracrine loop involving TNFα and IL8. Our findings reveal a novel mechanism in which neutrophils can increase the invasiveness of OSCC through the activation of invadopodia and matrix degradation.

Materials and Methods

Cell lines

UMSCC1 and UMSCC47 cells were from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). The UMSCC47 cell line is derived from a 53-year-old man with a metastatic lateral tongue squamous cell carcinoma (T3N1M0). UMSCC47 has a wild-type TP53 gene and is positive for HPV16 (20). UMSCC1 is derived from a man with a nonmetastatic floor of mouth squamous cell carcinoma (21). Both UMSCC1 and UMSCC47 were recently
shown to represent a unique genetic background and to be free of contamination (20). All cell cultures and experiments were performed in DMEM supplemented with 10% FBS, 100 nmol/L nonessential amino acids (Sigma), penicillin, and streptomycin (100 μg/mL). Peripheral blood neutrophils were isolated and purified from healthy donors as described before (22). Neutrophil viability by Trypan blue exclusion after 24 hours of incubation with cancer cells was 64.2% (±0.04%). The UMSCC1 and UM-SCC47 viability rates were 86.4% (±0.04%) and 83.7% (±0.08%), respectively, after 24 hours of incubation with neutrophils.

Antibodies and inhibitors

Cortactin antibody (Ab3333; mouse monoclonal) was from Abcam. Tks5 antibody (M-300, rabbit polyclonal) was from Santa Cruz Biotechnology. Rabbit P421 Cortactin antibody was purchased from Cell Signaling Technology. Secondary antibodies (goat anti-mouse Alexa Fluor-555 and goat anti-rabbit Alexa Fluor-647) were from Life Technologies. Anti-IL8 (ab18672) and TNFre (ab8348) antibodies were from Abcam. GM6001 inhibitor was from Sigma (M5939), and MMP9 inhibitor I was from Millipore (1177749-58-4).

Neutrophil conditioned media

Neutrophil conditioned media (CMN) was collected from a 24-hour cultures of neutrophils at a concentration of 400,000 cells/mL. Conditioned media from neutrophils and UM-SCC47 cocultures (CMC47) was collected from cultures of 800,000 neutrophils and 200,000 UM-SCC47 in 2 mL of DMEM for 24 hours. CMSEP (conditioned media from separated cultures) was collected from 100,000 UM-SCC47 or UM-SCC1 cells cultured on a 3.0-μm pore Transwell insert, physically separated from 400,000 neutrophils in the bottom chamber (total of 1 mL of media). For all the experiments, the media was centrifuged at 400 × g for 5 minutes to remove cells and stored at −80°C until use.

Matrix degradation and invadopodia analysis

Fifty thousand UM-SCC47 cells were plated on Alexa Fluor-488 gelatin matrix-coated Mattek dishes (10 mm) and incubated for 24 hours as described previously (23, 24). Where indicated, 50,000 (1:1) or 200,000 (4:1) human peripheral blood neutrophils were added to the bottom chamber. In direct coculture experiments, neutrophils were added to the upper chamber mixed with the cancer cells. Membranes were equilibrated at room temperature for 10 minutes before cells were added. The cells were allowed to invade for 24 hours followed by fixation in 3.7% PFA. Where indicated, 5 μg/mL of anti-TNFα or 10 μg/mL of anti-IL8 antibodies were added to the media 30 minutes before the experiment. Intact membranes were stained with Alexa Fluor-488 Phalloidin (Life technologies) for 30 minutes and visualized in a Nikon Eclipse TE300 epifluorescence microscope. The cells at the upper side of the membrane were removed using a Q-tip. Cell invasion was calculated as the average cell coverage area in μm² on the underside of the membrane compared with the UM-SCC47 control. Results were based on analysis of 20 fields (×20) in five independent experiments. The MMP inhibitor GM6001 (25 μmol/L) and MMP9 inhibitor (1 μmol/L) were added as indicated.

ELISA

ELISA kits for MMP9, EGF, and TNFre were purchased from Life technologies (Novex) and the human ELISA kit was custom made from Qiagen and included IL6, IL8, IL12, GM-CSF, TNFre, and TGFB. For the ELISA experiments, 200,000 UM-SCC47 cells were cultured in 2 mL of culture media. Where indicated, neutrophils were added at a 1:1 ratio (200,000) or 4:1 ratio (800,000). The cells were cultured for 24 hours and the supernatants were collected and centrifuged at 400 × g for 5 minutes to remove cells and stored at −80°C until use. The ELISA experiments followed the manufacturer’s protocol for each cytokine tested.

Statistical analysis

In experiments containing multiple group analysis, ANOVA was performed associated with Tukey tests. Where indicated, statistical analysis was calculated using the unpaired, two-tailed Student t test. Statistical significance was defined as P < 0.05. For all figures *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Error bars represent the standard error of the mean (SEM).
Results

Neutrophils increase the invasiveness of OSCC

We have analyzed the invasiveness of UMSCC47 and UMSCC1 cells in the presence of human neutrophils using a Transwell invasion assay. Both UMSCC1 and UMSCC47 cells invaded through Matrigel, although significantly more UMSCC47 cells invaded compared with nonmetastatic UMSCC1 cells (Supplementary Fig. S1A). Figure 1A illustrates the experimental design for the coculture invasion experiment. As seen in Fig. 1B and C, neutrophils increase the invasion of UMSCC47 cells and the effect is more significant with a high ratio (4:1) of neutrophils compared with a low ratio (1:1). UMSCC47 invasion is completely blocked by the MMP inhibitor GM6001, while the inhibition of MMP9 only partially blocks invasion. The experiment was repeated in the presence of 1 nmol/L of EGF as a chemoattractant in the bottom chamber, and similar results were observed (Fig. 1D). UMSCC47 viability after coculture was 83.7% (± 0.08%). Neutrophils viability was 64.2% (± 0.04%) after 24 hours of coculture, supporting earlier reports showing increased survival of TAN (25, 26). Together, these results show that direct coculture with neutrophils increases the invasiveness of cancer cells in the absence of any other stimulation.

Neutrophil-mediated increase in invasiveness does not require direct contact

In order to evaluate whether the increase in invasiveness required direct contact between cancer cells and neutrophils, we have used an adapted invasion assay. UMSCC47 cells were cultured in the upper chamber, whereas neutrophils were cultured in the lower chamber, thereby physically separating the neutrophils from the cancer cells (indirect coculture; Fig. 2A). Figure 2B shows that neutrophils significantly increase the invasiveness of UMSCC47 cells without direct contact. As seen in Supplementary Fig. S1B, UMSCC1 cells also increased invasion in the presence of neutrophils. These observations suggest that soluble secreted cues rather than direct contacts are necessary for the increase in invasion. GM6001 completely inhibits invasion, while MMP9 inhibitor partially blocks invasion.

Evidence of a feedback loop between UMSCC47 cells and neutrophils

We have used several variations of conditioned media to evaluate the possible feedback loop established between neutrophils and cancer cells, including conditioned media from neutrophils and UMSCC47 cells (CMC47), conditioned media from naive neutrophils only (CMN), and conditioned media from neutrophils and UMSCC47 or UMSCC1 cells physically separated by a Transwell membrane (CMSEP47 and CMSEP1). The conditioned media was added to the lower chambers of the invasion assay, as shown in Fig. 3A. CMC significantly increased the invasiveness of UMSCC47 cells (Fig. 3B). This is in contrast to conditioned media from naive neutrophils (CMN), which failed to increase the UMSCC47 invasiveness. Figure 3C shows that both CMSEP1 and CMSEP47 increased the invasion of UMSCC47 cells. Similar results were also observed with UMSCC1 cells (Fig. 3D).
The results show that the neutrophil-mediated increase in invasiveness depends on a costimulatory pathway between neutrophils and cancer that does not direct contact between them, suggesting a feedback loop involving secreted cues by cancer cells and neutrophils.

Neutrophils increase the number of invadopodia in UMSCC47 cells
Invadopodia are specialized subcellular structures used by cancer cells to invade surrounding tissues (17). We analyzed the number of invadopodia in UMSCC47 cells in the presence of
neutrophils. As shown in Fig. 4A, UMSCC47 cells show numerous invadopodia, identified by Tks5 and cortactin colocalization (19). A corresponding dot-like degradation of the matrix is seen in association with invadopodia. In neutrophil cultures or neutrophil and cancer cocultures, an ill-defined superficial degradation area was seen, corresponding to areas of neutrophil contact with the matrix (Supplementary Fig. S2A and S2B). Figure 4B shows that neutrophils significantly increase the number of invadopodia and the effect is more pronounced in the 4:1-ratio group compared with the 1:1-ratio group. Conditioned media experiments showed that CMC47 significantly increased the number of invadopodia in contrast to CMN (Fig. 4B). The size of the individual invadopodium was not changed by the presence of neutrophils (Fig. 4C). Cortactin phosphorylation is a key regulatory step of invadopodium maturation. Maturation of invadopodia requires the phosphorylation of cortactin at residues 421 and 466

![Figure 4](image-url)
Neutrophils increase UMSCC47 matrix degradation

Tumor invasion is a multistep process that requires tumor cells to degrade the basement membrane and remodel the surrounding connective tissue. We have analyzed the ability of UMSCC47 cells to degrade matrix in the presence of neutrophils or conditioned media. Fig. 5 shows that the presence of neutrophils increased UMSCC47 degradation, particularly at a 1:4 ratio of neutrophils and cancer cells. Also, similar to the results seen in the invasion experiments, conditioned media from UMSCC47 and neutrophils (CMC) increased matrix degradation while conditioned media from naïve neutrophils (CMN) failed to increase degradation.

TNFα and IL8 are increased in supernatants of neutrophil and UMSCC47 cocultures

Paracrine stimulation of cancer and immune cells has been reported in several malignancies, including breast adenocarcinoma (27). We investigated the possible role of secreted cytokines in the communication between UMSCC47 and neutrophils. Figure 6A shows that cocultures of UMSCC47 and neutrophils at a 4:1 ratio increased the secretion of TNFα in the culture media compared with neutrophils or UMSCC47 alone. Both UMSCC47 and neutrophils secrete MMP9, but there were no significant additive effects in the coculture groups (Fig. 6B). There was no significant change in the EGF concentration among the experimental groups (Fig. 6C). Figure 6D–G show the results from an ELISA experiment, where groups are tested for multiple cytokines simultaneously. Figure 6D shows that both neutrophils and cancer cells secrete IL8 in the supernatant. When these cells are cultured together, there is a sharp increase in the concentration of IL8 in the media, suggesting a costimulatory mechanism. UMSCC47 cells also secrete other cytokines, including TGFβ (Fig. 6E), IL6 (Fig. 6F), and GM-CSF (Fig. 6G). Importantly, TGFβ has been associated with a neutrophil protumor phenotype (2).

Neutrophil-induced IL8 and TNFα secretion was measured in the presence of neutrophils and UMSCC47 cells. Both the UMSCC1 and UMSCC47 cell lines used in this study showed an increase in invasion in the presence of neutrophils, but the nonmetastatic UMSCC1 cells were not used in further analyses because there were very few cells invading (Supplementary Fig. S1A). Importantly, these differences may also represent variations in the pathogenesis of the disease because UMSCC47 are HPV+ cells while UMSCC1 are HPV− carcinoma cell lines. HPV+ oral and oropharyngeal cancers show differences in clinical presentation, including younger age, higher N stage at presentation (lymph node metastasis), and overall improved survival compared with HPV− tumors (29). The differences seen in in vitro invasion in this study may represent the differences in the biologic and clinical differences between nonmetastatic UMSCC1 cells and metastatic UMSCC47 cells. Further studies are needed to clarify HPV+ and HPV− OSCC behavior in detail.

Here, we show for the first time that neutrophils increase the number of invadopodia and matrix degradation by oral cancer cells.
cells. Invadopodia have been identified in numerous invasive cancer cell lines (30), and evidence shows that invadopodia are associated with invasion and metastasis (31, 32). These specialized subcellular structures are activated by cortactin phosphorylation downstream of EGFR stimulation through the activation of Src and Arg kinases and combine focal degradation of the ECM with an actin-dependent protrusion (19, 23). Cortactin is required for invadopodia formation (33), and it converges different pathways leading to cancer invasion. The increase in cortactin phosphorylation reported here is similar to earlier observations by Dumitru and colleagues (16) in oropharyngeal cancers. The changes in matrix degradation induced by neutrophils are not as robust as the overall changes in invasion observed in the Transwell assays or invadopodia formation. This finding has two possible explanations: The thin matrix degradation assay used here analyzes one aspect of invadopodia activation, which is the localized recruitment of active MMPs to invadopodia. Because this is a thin matrix assay, it is very sensitive but lacks the ability of analyzing three-dimensional characteristics or stability of the invadopodia. The other explanation is that the function of invadopodia in invasion also includes actin-dependent protrusive forces, elongation, and adhesion, which could also be affected by neutrophils in our model. Thus, the degradation results must be analyzed in conjunction with the increase in number of invadopodia and cortactin phosphorylation, which collectively shows that neutrophils promote an invasive phenotype. It is also likely that the effects of neutrophils in OSCC involve not only increased invadopodia formation and matrix degradation but also changes in protein expression, motility, and cytoskeleton remodeling beyond invadopodia.

Our results point to a new paracrine activation loop between OSCC and neutrophils involving TNFα and IL8. A significant
In our experiments, MMP9 inhibition partially blocked the invasion and matrix degradation. Considering that both cells produce and secrete MMP9, there are two possible explanations for this. The first explanation is that neutrophil-secreted MMP9 directly remodels the matrix and facilitates invasion. This is unlikely because neutrophils did not invade through Matrigel or attach to the matrix. Neutrophil-associated degradation presented as a shallow, round area corresponding to the outline of neutrophils (Supplementary Fig. S2B). Neutrophils are washed away during fixation and therefore cannot be seen in the fixed samples. In addition, cancer cells migrate during the 24 hours of the experiment and can be seen close to or on top of neutrophil-degraded areas. The degradation "dots" caused by cancer cells are still visible under the neutrophil-mediated degradation, confirming the superficial nature of neutrophil degradation. Efficient invasion requires coordinated degradation as seen in the dot-like pattern typical of invadopodia-induced degradation. The second and more realistic possibility is that both UMSCC47-derived MMP9 at the invadopodia and neutrophil-secreted MMP9 can be used by cancer cells to promote invasion and focal matrix degradation. MMP9 can also indirectly signal to cancer cells by metabolizing other molecules, including TGFβ, IL1b, and TNFα (40). In our model, MMP9 inhibition partially blocked invasion and degradation but did not significantly change the concentration of TNFα or EGF in the supernatant (data not shown). Further studies will help clarify the role of MMP9 in oral cancer invasion.

In summary, our findings describe a novel mechanism linking neutrophils to OSCC invasion. This may represent a feedback loop between these two cells and a link between inflammatory states characterized by neutrophil infiltration and poor prognosis of squamous cell carcinoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: M.A.O. Magalhaes
Development of methodology: C.X. Sun, M.A.O. Magalhaes
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Glogauer, C.X. Sun, G. Bradley, M.A.O. Magalhaes
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.E. Glogauer, C.X. Sun, M.A.O. Magalhaes
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