Effects of *Fusobacterium nucleatum* on migration and cytokines production of AGS gastric adenocarcinoma cell line

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**Abstract.** Gastric cancer (GC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer death worldwide. *Helicobacter pylori* (*Hp*) infection is an important risk factor for GC. However, the etiology of the tumor is multifactorial, since only 1-3% of infected patients develop cancer. Therefore, attention should be focused on the role of microbiota in gastric tumorigenesis since in some studies an alteration of the microbiota in GC has been shown. *Fusobacterium nucleatum* (*Fn*) has been found in biopsies of patients with GC. However, since its role is not clearly established, this study investigated the effects of *Fn* infection on the human gastric adenocarcinoma cell line AGS. Our results showed that *Fn* co-localized at level of the plasma membrane demonstrating the ability of *Fn* to adhere to AGS cells. In addition, increases in incubation times were associated with its intra-cellular localization with loss of the classic curved rod shape. Interestingly, *Fn* determined a greater capacity of cell migration compared to untreated AGS cells. Moreover, IL-4 expression significantly increased in *Fn* infected GC cells. Since cancer cell migration is an integral component of the metastatic process, additional studies are needed to better understand the mechanisms underlying the *Fn*/host interaction.

**Keywords:** *Fusobacterium nucleatum*, gastric cancer, IL-4 production.

**INTRODUCTION**

Gastric cancer (GC) is the third leading cause of cancer deaths worldwide (Ferlay et al., 2021) since most patients are diagnosed with an advanced stage of disease. Thus, great efforts are made on tumor prevention and early diagnosis. *Helicobacter pylori* (*Hp*) infection is the major risk factor for the onset
and evolution of GC, but effectiveness of its eradication by drug therapies is still discussed (Piscione et al., 2021). Actually, it is not clear whether the presence of Hp or the imbalanced gastric microbiota generates the conditions for Hp or other pathogens colonization. *Fusobacterium nucleatum* (Fn), involved in periodontal disease, has been detected also in human tumor samples (Han et al., 2015). Indeed, several studies showed the presence of Fn in colorectal adenomas (Gethings-Behncke et al., 2020) and in primary colorectal cancer as compared to healthy tissue (Kim et al., 2020). These data suggest that Fn plays a role in the initiation and progression of colorectal tumorigenesis (Abed et al., 2017). Fn has also been isolated in esophageal (Yamamura et al., 2017) and gastric cancer (Hsieh et al., 2018). In the intestine Fn alters the cellular pathways through FadA, an adhesin that stimulates the proliferation of host cells and regulates inflammatory response (Sahan et al., 2018). Elevated levels of Fn were detected in biopsies of patients suffering from GC, associated with low levels of Hp (Hsieh et al., 2018). Hp colonizes the gastric mucosa through its virulence factors and, by urease enzyme, it raises the gastric pH, making the stomach a more permissive environment towards other pathogens such as Fn (Servetas et al., 2016; Hsieh et al., 2018). Periodontal disease has a link with GC (Zhou et al., 2018) and Fn could play a key role in making periodontal disease a worsening factor for the development of GC (Flemer et al., 2018; Toma et al., 2018). Therefore, the aim of this study was to evaluate the effects of Fn infection on a model of human gastric adenocarcinoma cell line AGS in order to verify whether it affects metastatic behavior.

**MATERIALS AND METHODS**

**Bacterial culture**

*Fusobacterium nucleatum* (ATCC 25586) was cultured as previously reported (Maccelli et al., 2020). Bacteria were harvested by centrifugation at 6000 \( \times \) g for 10min at 4°C. The bacterial pellets were washed twice, resuspended with phosphate-buffered saline and quantified using a microplate reader.

**Cell line culture**

Human gastric adenocarcinoma AGS cell line (Cell Lines Services, Epplheim, Germany) was cultured as previously described (Savino et al., 2022). AGS cells were infected with Fn at multiplicity of infection (MOI) of 100-2000. Supernatants were collected after 24h post-infection and used in Luminex Assay.

**Confocal microscopy**

AGS cells were grown on glass coverslips and infected with Fn at different MOI. At the established time they were fixed (2% paraformaldehyde for 10min), treated for 60min with 10% Goat Serum and incubated with primary antibody anti-Fn ANT0084 (DIATHEVA, Italy) at 1:100 dilution. Secondary antibody (Alexa Fluor 488 conjugated, Thermo Fisher Scientific, USA) was used at 1:200 dilution. Then, the cells were treated with PHK26 (SIGMA ALDRICH, USA) at 1:1000 dilution to stain AGS cells membrane. Images were obtained using Zeiss LSM800 confocal microscopy and acquired by Zen Blue software (Carl Zeiss, Germany).

**Cell migration assay**

Cells were infected with Fn at a MOI 500-1500 and wound healing scratch assays were performed using noninfected AGS cells as control. Cells were cultured to 95% confluence in 24-well plates and after 24h, a scratch wound was made followed by Fn infection. Cellular migration was recorded after 24h and 48h.

**Detection and quantification of cytokines with Luminex assay**

Expression levels of IFN-γ, IL-1b, IL-4, IL-6, IL-10, IL-17A were determined in AGS cell culture supernatants using Human kits (Milliplex, Merck Millipore, USA) and Luminex MAGPIX detection system following the manufacturer’s instructions. The levels of cytokines (pg/ml) were estimated using a 5-parameter polynomial curve (Bio-Plex Manager Software, Bio-Rad, USA).

**Statistical analysis**

The data are reported as the representative values of three independent experiments.

**RESULTS**

**Localization of Fn after infection of AGS cells by confocal microscopy**

Fn localization and its effects on AGS cells morphology were evaluated using a confocal microscope. In Figure 1 orange fluorescence locates AGS cell membrane, while green fluorescence identifies Fn as detected using a specific antibody. After 24h, polygonal AGS cells were
Effects of *Fusobacterium nucleatum* on migration and cytokines production of AGS gastric adenocarcinoma cell line

Surrounded by rod-shaped bacteria. Interestingly, signals deriving from bacteria in intracellular compartment appeared with a cytoplasmic spotted pattern (Figure 1A–C). In addition, some AGS cells showed a yellow fluorescence signal, deriving from *Fn* co-localization with AGS cell membrane (Figure 1B and D).

**Figure 1.** Localization of *Fn* after infection of AGS cells by confocal microscopy. Representative images of co-culture of *Fn* (MOI 100) and AGS cells acquired using confocal microscopy (green and orange fluorescence respectively). Images were captured near the cells contact to coverslip side (A to C) or multiple focal plane (Z stack) to obtain XZ, YZ projection (D). Bar=10µm

Effects of *Fn* infection on migration and cytokine expression in AGS cells

Acquisition of migratory properties is a prerequisite for cancer progression. *Fn* modulates migration and immune cell signaling in colorectal cancer (Casasanta MA et al., 2020).
Cell migration of AGS cells was assessed using a wound healing assay after 24h of infection. Our results (Figure 2) demonstrated that \(Fn\) infection promoted cell migration, as compared to AGS untreated cells.

To find out whether \(Fn\) was able to induce the secretion of cytokines from these cells, the expressions of IFN-\(\gamma\), IL-1\(\beta\), IL-4, IL-6, IL-10, IL-17A were analyzed in the supernatants of AGS cells collected after 24h of co-culture with \(Fn\). Interestingly, while IFN-\(\gamma\) secretion increased at MOI 1000 as compared to control cells, \(Fn\) infection increased IL-4 and IL-6 from MOI 1000 to MOI 2000, as compared to uninfected cells (Figure 3).

**DISCUSSION**

The present study showed that \(Fn\) co-culture of AGS cells caused an increase in cell migration capacity and induced IL-4 and IL-6 secretion. It is known that IL-4 plays an important role in cancer cell proliferation and migration enhancing the malignant phenotype (Hallett et al., 2012). Moreover, increased plasma IL-4 levels were significantly higher in GC patients than in healthy controls (Cardenas et al., 2018). The preliminary results obtained in this study suggest that IL-4 could increase the migration ability of AGS cells. Further analysis will be needed to verify this hypothesis. Moreover, we demonstrated the adhesion of \(Fn\) to the plasma membrane of AGS cells and its presence in their cytoplasm. These data are in agreement with the evidence obtained in colorectal adenocarcinoma cells in which the bacterium is internalized by tumor cells (Casasanta et al., 2020). In conclusion, we hypothesize that future studies characterizing how \(Fn\) influences signaling pathways during cancer development will lead to targeted approaches that could potentially decrease the risk of progression to gastric cancer.

**REFERENCES**


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