An active product of cruciferous vegetables, 3,3′-diindolylmethane, inhibits invasive properties of extravillous cytotrophoblastic cells

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Abstract

OBJECTIVES: During implantation, human trophoblastic cells have to proliferate, migrate and invade pregnant uterus. A natural product of cruciferous vegetables, 3,3'-diindolylmethane (DIM), is known to induce some stress response genes (such as glucose-regulated protein 78 kDa (GRP78)) and to have anti-invasive and pro-apoptotic effects on tumor cells. Therefore, we have investigated the potential effect of DIM on invasive extravillous cytotrophoblasts (evCTBs) cells.
MATERIALS AND METHODS: evCTBs were purified from first trimester trophoblasts and cultured in presence or not of DIM for 48h. In order to evaluate invasive properties of cells, they were seeded on collagen-coated insert following boyden chamber principle and matrix metalloproteinases (MMPs) and GRP78 expression was evaluated by qPCR.
RESULTS: We showed that DIM decreases (p=0.013) invasive properties of evCTBs. In parallel, we determined that MMP-2, -7 and -9 which are involved in evCTBs invasion and known to be regulated by DIM, are not affected by DIM in evCTBs. In contrast, MMP-1 mRNA is induced (p=0.03) and MMP-12 is decreased (p=0.01) in DIM treated cells. Moreover, DIM treatment does not affect GRP78 mRNA expression in evCTBs.
CONCLUSIONS: Collectively, the present results provide evidence that DIM does not impact evenly on evCTBs and cancer cells.

ORIGINAL ARTICLE

INTRODUCTION

To allow embryo implantation, trophoblast cells of the human placenta have to proliferate, migrate and invade pregnant uterus in a way that is very similar to cancer cells. Extravillous cytotrophoblastic cells (evCTBs) form clusters of proliferating cells (proliferating evCTBs) (Lacroix *et al.* 2005). As they further differentiate, evCTBs lose the ability to divide within cell columns and become mobile and highly invasive (invading evCTBs). Amongst the invading evCTBs, interstitial cells invade the decidualized endometrium and the proximal third of the myometrium (interstitial invasion), whereas other cells (endovascular evCTBs) invade the uterine spiral arteries (endovascular invasion) (Lunghi *et al.* 2007). Defective trophoblastic invasion can lead to abortion or different obstetrical syndromes such as preeclampsia, intrauterine growth restriction, and preterm labor (Norwitz 2006). In con-

trast, excessive trophoblastic invasion often results in deficient development of the decidua with abnormal attachment of the placenta (Bauer & Bonanno 2009). Exaggerated trophoblastic invasion is also a major feature of gestational trophoblastic diseases such as hydatidiform moles (Wells 2007). Trophoblastic invasion is rendered possible due to expression of specific cell adhesion molecules, matrix-digesting proteases and proto-oncogene, and activation of telomerase. But it is tightly regulated both in time (first trimester of pregnancy) and in space (limited to the endometrium and to the proximal third of the myometrium) (Pijnenborg *et al.* 1980).

Indole-3-carbinol (I3C) which is produced by members of the Cruciferae family could have a protective effect against cancer since it induces apoptosis in tumor cells (Chinni *et al.* 2001; Choi *et al.* 2010; Hong *et al.* 2002). This compound might have also developmental toxicity (Wilker *et al.* 1996). Nevertheless, Yan *et al.* contradicted this finding while suggesting that I3C could induce placental CYP1A1 and thus intensify fetal growth-retardation produced by prenatal tobacco-smoke in rats (Yan *et al.* 2006).

I3C is converted under acidic conditions to a series of oligomeric products among which 3,3'-diindolylmethane (DIM) is a major component. These products are responsible for its biological effects *in vivo* (Bradlow & Zeligs 2010; De Kruif *et al.* 1991). DIM exerts different effects which are of benefit against cancer. Indeed, it was found to induce some stress response genes including glucose-regulated protein 78 kDa (GRP78) (Sun *et al.* 2004) which has been recently suggested as an important regulator of trophoblastic cell invasion (Arnaudeau *et al.* 2009). DIM could also be responsible for decreased expression of matrix metalloproteinases -9 (MMP-9) and -2 (MMP-2) and cathepsin D in different type of cells (Hung & Chang 2009; Kong *et al.* 2007; Meng *et al.* 2000; Rajoria *et al.* 2011). These proteases play a key role in digestion of matrix components and are necessary for trophoblastic invasion of pregnant uterus (Cohen & Bischof 2007).

Since evCTBs behave like cancer cells to allow embryo implantation, we have examined in the present report the effects of DIM on evCTBs invasiveness.

MATERIAL AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), Hanks balanced salted solution (HBSS), trypsin-EDTA and gentamicin were products of Invitrogen (Basel, Switzerland). Fetal bovine serum (FBS) was from Biochrom AG (Oxoid AG, Basel, Switzerland). Wst-1 cell proliferation assay and DNase I were purchased from Roche (Diagnostics GmbH, USA). Rat tail collagen type I and collagenase were from Sigma (Sigma-Aldrich, Buchs, Switzerland). DIM was from Enzo Life Sciences (Lausanne, Switzerland).

Cell culture

Trophoblastic tissues were obtained from patients undergoing a legal abortion during the first trimester (8–12 weeks of gestation). Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local ethics committee. CTB were isolated from first trimester placentas as described elsewhere (Bischof *et al.*, 1995). In brief, fresh tissue specimen were isolated and washed several times in sterile HBSS. Tissue was then enzymatically digested 5 times for 20 min at 37 °C (0.25% trypsin, 0.25 mg/ml DNase I). After incubation, the trypsin cocktail was neutralized with FBS, and the cells resuspended in DMEM. This cell suspension was filtered on 50 µm mesh and seeded on Petri dishes for 10 min. Supernatants containing evCTBs were centrifuged and cells were resuspended in culture medium and seeded in 6-well plates (4×10^6 cells per well). The next day, evCTBs were treated or not with 20 µM DIM for 48 h before being harvested for qPCR, proliferation or invasion assay.

Proliferation assay

evCTBs were seeded at a density of 10^5 cells in 96-well plate. Wst-1 cell proliferation assay was used according to the manufacturer's protocol. Absorbance was recorded at 450 nm after 60 min using a 96-well plate reader.

Invasion assay

Cell invasion assay was performed in an invasion chamber based on the Boyden chamber principle as previously described (Arnaudeau *et al.* 2009).

RNA extraction

evCTBs (5×10^6 cells) were cultured in 6-well plate for 48 h before total RNA was extracted using RNeasy Mini kit (QIAGEN, Basel, Switzerland) following manufacturer's instructions.

Real-time quantitative reverse transcription-PCR

Reverse transcription was performed with 400 ng of total RNA in a final volume of 20 µl using QuantiTect Reverse Transcription kit (QIAGEN, Basel, Switzerland). The quantitative detection of the PCR product was performed using the qPCR Mastermix Plus for SYBR Green I (Eurogentec, Seraing, Belgium), supplemented with fluorescein (Bio-Rad, Reinach, Switzerland), with the iCycler iQ System (Bio-Rad). The relative expression was normalized to the housekeeping gene cyclophilin A. Oligonucleotide primers for qPCR were as follows: human cyclophilin A forward 5'-TACGGGTCCTGGCATCTTGT-3' and reverse 5'-CCATTTGTGTTGGGTCCAGC-3', human GRP78 forward 5'-CGTGGAGATCATCGCCAAC-3' and reverse 5'-ACATAGGACGGCGTGATGC-3', human MMP-1 forward 5'-AAAGACAGATTCTACATGCG -3' and reverse 5'-TGCTTCACAGTTCTAGGGA-3',

human MMP-2 forward 5'-ATAACCTGGATGCCGTCGT-3' and reverse 5'-AGGCACCCTTGAAGAAGTAGC-3', human MMP-7 forward 5'-CCAGATGTTGCAGAATACTC-3' and reverse 5'-CCACTGTAATATGCGGTAAG-3', human MMP-9 forward 5'-CTGAGAACCAATCTCACCGACA-3' and reverse 5'-AGATTTCGACTCTCCACGCA-3' and human MMP-12 forward 5'-CCAGCTCTCTGTGACCCCAA-3' and reverse 5'-TCCCACGGTAGTGACAGCATC-3'.

Statistical analysis

Data were expressed as means ± SEM for 3 independent experiments run in triplicate. Statistical differences between samples were assessed by the Student's t test and the p-value <0.05 was considered significant.

RESULTS AND DISCUSSION

Epidemiologic studies have demonstrated a correlation between fruits and vegetables consumption and the decreased risk of cancer. The experimental explanation of this observation derives from the identification of compounds with cytoprotective effect on normal cells and noxiousness effect on cancer cells, such as I3C and its biologically active derivatives, and particularly DIM (Higdon *et al.* 2007). They act on different signaling pathways involved on cell cycle arrest, angiogenesis, invasion, metastasis and epigenetic behavior of cancer cells (Banerjee *et al.* 2011).

In pregnant uterus, during the first trimester of pregnancy, non malignant evCTB behave like tumor cells to allow embryo implantation. An abnormal trophoblastic invasion can lead to different obstetrical syndromes (abortion, preeclampsia, intrauterine growth restriction, choriocarcinoma, placenta accreta…). During this crucial placentation step, DIM compounds could decrease invasive properties of trophoblastic cells, as observed on cancer cells. We thus evaluated, DIM effects onevCTBs invasiveness.

Effect of DIM on invasive properties of evCTBs

evCTBs have to invade pregnant uterus to allow embryo implantation and placentation. Compounds which can lower the ability of these cells to invade can presumably effectively affect embryo implantation or feto-placental exchanges. It has already been observed that DIM has anti-invasive properties against several cancer cells (Rahimi *et al.* 2010; Rajoria *et al.* 2011). Thus, we wanted to evaluate its effect on the invasive properties of evCTBs. As shown in Figure 1, treatment of evCTBs with 20 µM of DIM leads to a significant (p=0.013) decrease of their invasive properties (~30%) when compared to control cells (set at 100%). A cytotoxicity assay was performed in parallel of invasion experiments. At this concentration, DIM does not affect cytotoxicity of evCTB (data not shown) suggesting that effect of DIM on evCTB invasiveness is not due to general cytotoxicity.

Effect of DIM on MMPs mRNA expression

MMPs are reliable markers of cell invasion in general. The MMP-2 and -9 are the most studied in first trimester CTBs (Isaka *et al.* 2003; Staun-Ram *et al.* 2004) but there are also many other MMPs found in placenta and involved in trophoblast invasion (Cohen *et al.* 2006; Vettraino *et al.* 1996). Indeed, MMP-1 has the property to cleave fibrillar collagens within their triple helix at neutral pH and serves an essential role in initiating efficient matrix turnover (Pardo & Selman 2005). MMP-7 has a broad capacity to degrade matrix components including elastin, fibronectin and proteoglycans (Cohen *et al.* 2006). MMP-12 was recently identified as another important mediator of uterine vascular remodeling during pregnancy (Harris *et al.* 2010). Together, these MMPs could cooperate to degrade main extracellular matrix components of the uterus and could be essential for human placentation. Since DIM has been already shown to target MMPs in estrogen responsive cancers (Rajoria *et al.* 2011), we have determined the effects of this compound on MMPs mRNA expression. In contrast to what was observed in thyroid cancer cell lines (Rajoria *et al.* 2011), DIM does not decrease mRNA expression of MMP-2 and -9 in evCTBs (Figure 2A). In contrast, DIM slightly but significantly (p=0.01) decreases expression of MMP-12 (Figure 2B). Surprisingly, it also significantly (p=0.03) increases expression of MMP-1 (Figure 2C) and tends to induce expression of MMP-7 (Figure 2D). Globally, the effect of DIM on inhibition of invasive properties of evCTBs cannot be directly explained by its impact on MMPs mRNA expression.

Effect of DIM on GRP78 mRNA expression

DIM has already been shown to activate cellular stress response pathways *in vitro*. Among its target genes, GRP78 mRNA was significantly increased in DIM-treated breast and prostate cancer cells compared to

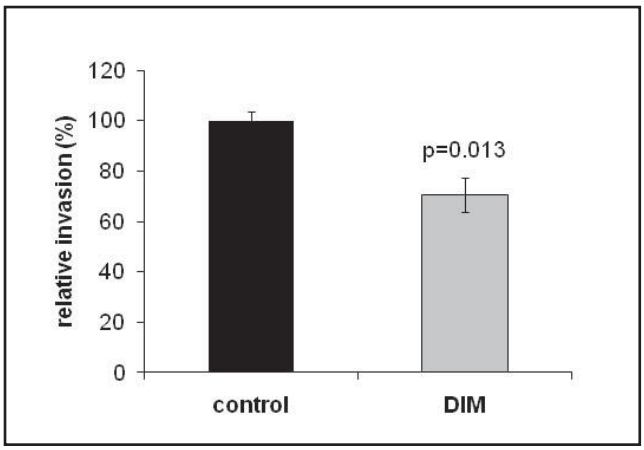

Fig. 1. Effect of DIM on evCTBs properties. evCTBs were cultured for 48 h ± 20 µM of DIM before invasion assay was performed. Results are expressed as percent of untreated (control) cells.

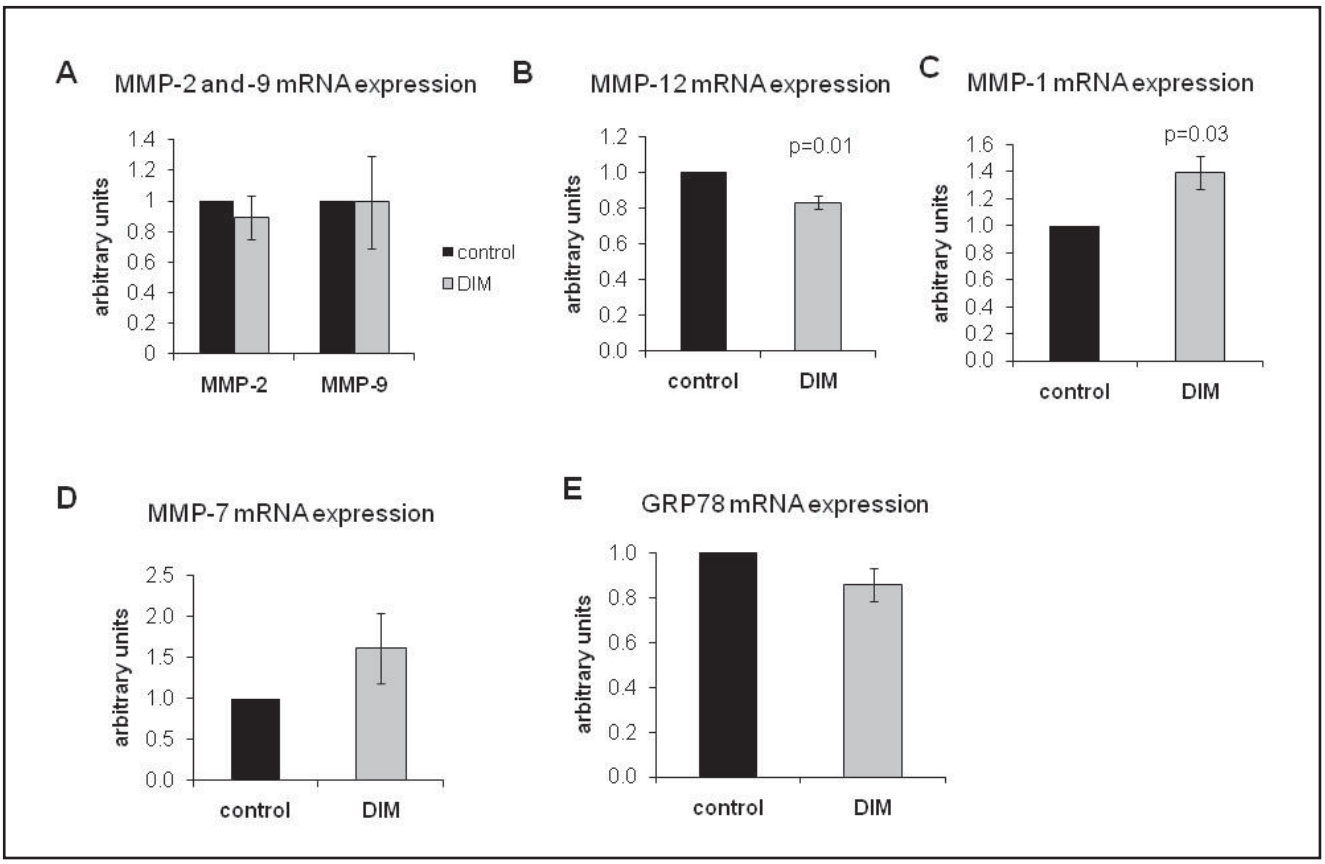

Fig. 2. Effect of DIM on MMPs and GRP78 mRNA expression of evCTBs. evCTBs were cultured for 48 h ± 20 μM of DIM. Quantitative analysis of MMP-2 and MMP-9 (**A**), MMP-12 (**B**), MMP-1 (**C**) MMP-7 (**D**) and GRP78 (**E**) mRNA was performed by RT-qPCR. Results reference set at 1 correspond to untreated (control) cells.

untreated cells (Sun *et al.* 2004). We have recently suggested that GRP78 could play an important role in regulating trophoblastic cell invasion (Arnaudeau *et al.* 2009). To determine whether this gene is also upregulated by DIM in evCTBs, we have used RT-qPCR to quantify GRP78 mRNA. As shown in figure 2E, mRNA expression of GRP78 is not induced in DIM-treated evCTBs compared to control cells. In these non malignant invasive cells, and in contrast to malignant cells, DIM does not induce expression of GRP78.

In conclusion we reported that DIM significantly decreased evCTB invasiveness *in vitro*, as reported on cancer cells. This observation leads us to think about its effects on pregnancy, especially for women with high risk of preeclampsia development and/or intrauterine fetal growth restriction. The mechanisms by which DIM could decrease invasive properties of evCTBs seem to be different as those already described in cancer cells, and still to be elucidated.

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