Diacylglycerol Kinase is Required for HGF-induced Invasiveness and Anchorage-independent Growth of MDA-MB-231 Breast Cancer Cells

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Abstract. *Background: Estrogen receptor (ER)-negative breast cancers have a worse prognosis than ER-positive cancers, being more aggressive and overexposed to stimuli leading to their progression. Hepatocyte growth factor (HGF) has been associated with proliferation, migration and invasion of tumor cells, and several tumors, including those of breast cancer, produce HGF and overexpress its receptor. Diacylglycerol kinases (Dgks), which phosphorylate diacylglycerol to phosphatidic acid, are key regulators of cell signaling. Our research was focused on their role in HGF-induced invasion of MDA-MB-231 cells, a model of ER-negative breast cancer. Materials and Methods: Dgk activity was evaluated with a kinase assay, MDA-MB-231 cell invasion via culturing of cells in matrigel-coated transwells, and anchorage-independent growth was assessed using a soft agar assay. Results: HGF induces Dgk activation in MDA-MB-231 cells that is required for cell invasiveness. Moreover, Dgks are involved in MDA-MB-231 anchorage-independent growth. Conclusion: Dgks could be a target for ER-negative breast cancer therapy.*

Breast tumors expressing estrogen receptors (ER) are generally responsive to therapeutic strategies using selective ER modulators like tamoxifen and have a better prognosis than ER-negative ones (1). ER-negative breast cancer represents about 30% of invasive breast cancer and is more aggressive (2). The ER-negative tumors, unresponsive to anti-estrogens, are often overexposed to stimuli leading to their progression.

Hepatocyte growth factor (HGF) plays a well-known role in the process of tumor invasion and metastasis. HGF stimulates proliferation, dissociation, migration and invasion in a wide variety of tumor cells, and is a potent angiogenic factor (3, 4). Stromal fibroblasts are the main source of HGF, however, several tumor cells have been shown to produce HGF (5, 6). HGF elicits its biological functions through binding to its specific receptor, c-Met. The role of HGF and c-Met in human cancer metastasis is well established: the presence of c-Met has been reported in tumors of the thyroid, ovary, pancreas, breast, prostate and gastrointestinal tract (7-10) and its level of expression has been correlated with tumor progression and poor outcome in breast cancer patients (11, 12). In addition, c-Met has also been reported to be an independent prognostic factor in breast cancer (13).

Diacylglycerol kinases (Dgks), which phosphorylate diacylglycerol to generate phosphatidic acid (PA), comprise a family of ten distinct enzymes, grouped in five classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich C1 domains (14). It has been shown that Dgk-α, an isoform of class I Dgk, is activated by several growth factors: HGF and VEGF in epithelial and endothelial cells (15, 16), and IL-2 activation in T-cells (17). Inhibition of Dgk-α activity, obtained either pharmacologically or by expression of dominant negative mutant or by RNA interference, impairs

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HGF-, VEGF- and ALK-induced chemotaxis and proliferation in several cell types (15, 16, 18), as well as angiogenesis in endothelial cells (16). Similarly, in T-cells, pharmacological inhibition of Dgk-α severely impairs IL-2-induced G1- to S-phase transition (19).

Here we investigated whether HGF stimulation could induce activation of class I Dgks in the MDA-MB-231 breast cancer cell line and whether Dgk is involved in the invasiveness of MDA-MB-231 cells and their anchorage-independent growth.

Materials and Methods

Reagents. Cell culture medium and reagents were from Gibco (Invitrogen, Carlsbad, CA, USA), recombinant HGF was from Peprotech (London, UK), class I Dgk pharmacological inhibitor R59949 was from Sigma (St. Louis, MO, USA). All reagents were from Sigma, unless otherwise indicated.

Cell cultures. MDA-MB-231 (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco), penicillin (100 u/ml), streptomycin (100 μg/ml) and an antimycotic (0.25 μg/ml).

Dgk assay. Cells were starved in 0.2% FCS for 24 h, treated with 100 ng/ml HGF for 15 min and homogenised with a buffer containing 25 mM Hepes (pH 8), 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM ammonium molybdate, 10 mM NaF, 1 mM sodium orthovanadate and Protease Inhibitor Cocktail. Homogenates were collected, passed through a 23 G syringe and centrifuged at 500 xg for 15 min at 4˚C. Protein concentration was determined using the BCA method (Pierce, Rockford, IL, USA). Homogenates were incubated for 5 min with a saturating substrates concentrate (1 mg/ml diolein, Fluka), 5 mM ATP, 3 μCi/μl (γ^{32}P)-ATP (GE Healthcare), 10 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 mM EGTA in 25 mM Hepes (pH 8) in the presence or absence of 1 μM R59949. Lipids were extracted as described elsewhere (20), and PA was separated using TLC in chloroform: methanol:water: 25% ammonium hydroxide (60:47:11:4). TLC plates had been previously coated with potassium oxalate 1.3%, EDTA 5 mM:methanol (3:2) and dried. (^{32}P)-PA was identified by co-migration with non-radioactive PA standards stained by incubation in an iodine chamber. Radioactive signals were detected and quantified with the GS-250 Molecular Imager and Phosphor Analyst Software (Bio-Rad, Hercules, CA, USA).

Cell invasion assay. MDA-MB-231 cells were plated in the upper part of a transwell, the surface of which was coated with Matrigel (BD Biosciences, Erembodegem, Belgium). The lower chamber was filled with DMEM 0.2% FCS in the presence or absence of 1 μM R59949. After 15 min, 50 ng/ml HGF were added and cells were incubated for further 16 h. Non-migrated cells were removed from the upper part of the filters, while cells which migrated through the Matrigel in the lower part of the transwell were stained with Diff-Quik (Baxter, Deerfield, IL, USA) and counted.

Soft agar assay. MDA-MB-231 cells were suspended in 0.35% agar in DMEM 1% FCS and then plated (5x10^4 cells/well, 12-well plate) on a layer of 0.7% agar in DMEM 1% FCS in presence of 1 or 10 μM R59949. Upon 20 days of treatment, the colonies of living cells were stained with MTT and counted with the Quantity One software (Bio-Rad).

Statistical analysis. Where appropriate, data are presented as the mean±SEM and the statistical significance was assessed using Student's *t*-test.

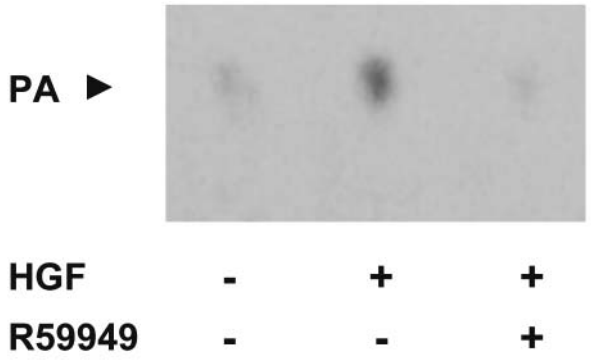

Figure 1. *HGF activation of Dgk in MDA-MB-231 breast cancer cells (representative TLC plate) (^{32}P)-phosphatidic acid (PA) was separated using TLC and identified by co-migration with non-radioactive PA standards. HGF: hepatocyte growth factor; R59949: Dgk inhibitor.*

Results

HGF induced the activation of class I Dgk in the MDA BD-231 breast cancer cell line. The MDA-MB 231 human breast cancer cell line is often used as a model of ER-negative breast cancer. This cell line is considered particularly suitable for pre-clinical studies since it is highly aggressive both *in vitro* and *in vivo* (21).

As we previously demonstrated that HGF activated Dgk-α in epithelial cells (15), we investigated whether HGF would induce the activation of Dgk in MDA-MB-231 breast cancer cells (Figure 1). Indeed, 100 ng/ml HGF stimulated the activation of Dgk, as measured by its specific kinase activity *in vitro*. The Dgk activity up-regulated by HGF was inhibited when assayed in the presence of 1 mM R59949, a pharmacological inhibitor of class I Dgks.

HGF-induced invasiveness of the MDA-BD-231 breast cancer cell line. One of the peculiarities of aggressive tumors is their ability to metastasize. MDA-MB-231 cells, both spontaneously and upon growth factor stimulation, are able *in vitro* to pass through a matrigel layer, mimicking their ability to invade extracellular matrices *in vivo* (22).

In order to provide evidence that Dgk may be involved in the HGF-induced invasiveness of MDA-MB-231 cells, we investigated whether the pharmacological inhibition of Dgk impairs HGF invasive activity in these cells. Indeed, 50 ng/ml HGF in the lower chamber of the transwells promoted the

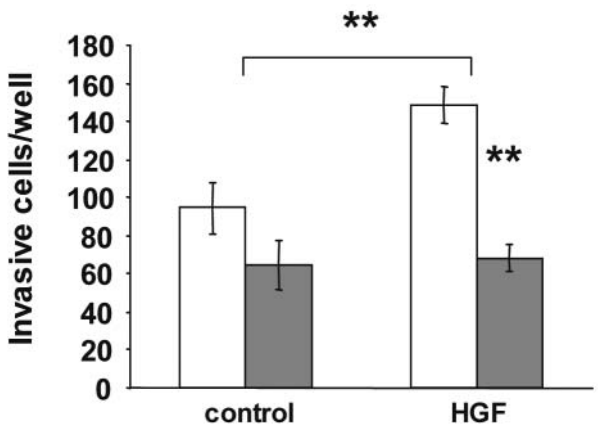

Figure 2. *HGF-induced invasion of MDA-MB-231 cells, as shown using a Matrigel assay. Empty bars: without R59949, solid bars: with R59949. **p<0.01 vs. control.*

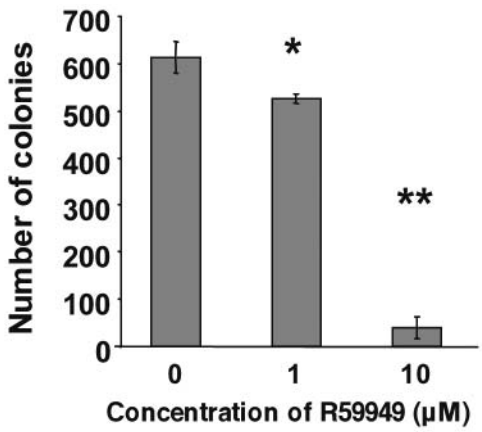

Figure 3. *Soft agar growth of MDA MB 231 breast cancer cells (MTT assay). **p<0.01 and *p<0.05 vs. control.*

invasion of MDA-MB-231 through a matrigel layer, while pretreatment with 1 μM R59949 for 15 min abolished the HGF-induced invasive capability of the cells (Figure 2).

Soft agar growth of the MDA BD-231 breast cancer cell line. The soft agar assay evaluates the ability of cells to form colonies in the absence of adhesion in a semi-solid medium, a feature of neoplastic cells. MDA-MB-231 cells are able to form colonies in soft agar when cultured in 1% FCS. Simultaneous treatment with 1 μM or 10 μM R59949 reduced the number of colonies formed by ~15% and 95% respectively (Figure 3).

Discussion

Treatment of breast cancer with selective ER modulators is an example of a successful therapy targeting estrogen receptor expression (23). However, its efficacy is limited to ER-positive breast tumors, which generally have a better prognosis (1) than ER-negative tumors. Indeed, ER-negative breast tumors are more aggressive (2), although the processes determining local invasion and the formation of metastases, responsible for their aggressiveness, are not completely understood at the molecular level. Revealing the molecular pathways involved in ER-negative hormone-independent breast cancer progression and metastasis may offer suitable targets for the development of new efficient anticancer therapies.

In recent years, diacylglycerol kinases have been intensively investigated either as negative or positive regulators of cell signaling. It has been shown, for instance, that activation of Dgk-α is required for growth factor-induced proliferative and chemotactic signaling (15-18), as well as for negative feedback in TCR signaling (24, 25).

Here we showed that HGF stimulation induced the activation of class I Dgks in MDA-MB-231, an ER-negative human breast cancer cell line considered particularly suitable for pre-clinical studies since it is highly aggressive both *in vitro* and *in vivo*. HGF-induced Dgk activation in turn mediated the passage of MDA-MB-231 cells through a matrigel layer, a peculiarity of aggressive cancer cells which mimics the invasion of extracellular matrices and the ability of these cells to metastasize; the pharmacological inhibition of Dgk activity abolished the effects elicited by HGF. Moreover, we showed that Dgk was involved in the anchorage-independent growth of MDA-MB-231 cells, a typical feature of tumor cells, as the inhibition of Dgk activity drastically reduced the number of colonies formed in soft agar.

These results fully demonstrate the biological relevance of Dgk in signaling pathways leading to cell migration elicited by growth factors, and suggest that class I Dgks could be a suitable target for the development of efficient therapies of ER-negative breast cancer.

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