MOLECULAR ONCOLOGY 9 (2015) 513–526

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

Increased extracellular pressure stimulates tumor proliferation by a mechanosensitive calcium channel and PKC-β

Marc D. Basson[a,*], Bixi Zeng[a], Christina Downey[a], Madhu P. Sirivelu[a], Jetze J. Tepe[b]

[a]Department of Surgery, Michigan State University College of Human Medicine, 1200 E Michigan Ave, Lansing Charter Township, MI 48912, USA

[b]Department of Pharmacology, Michigan State University, 1355 Bogue Street, B440 Life Sciences Building, East Lansing, MI 48824, USA

ARTICLE INFO

Article history:
Received 2 September 2014
Received in revised form
17 October 2014
Accepted 20 October 2014
Available online 23 October 2014

Keywords:
Calcium channels
Cav3.3
PKC
NF-kB
Pressure
Proliferation

ABSTRACT

Large tumors exhibit high interstitial pressure heightened by growth against the constraining stroma. Such pressures could stimulate tumor proliferation via a mechanosensitive ion channel. We studied the effects of 0–80 mmHg increased extracellular pressure for 24 h on proliferation of SW620, Caco-2, and CT-26 colon; MCF-7 breast; and MLL and PC3 prostate cancer cells, and delineated its mechanism in SW620 cells with specific inhibitors and siRNA. Finally, we compared NF-kB, phospho-IkB and cyclin D1 immunoreactivity in the high pressure centers and low pressure peripheries of human tumors. Pressure-stimulated proliferation in all cells. Pressure-driven SW620 proliferation required calcium influx via the T-type Ca^{2+} channel Cav3.3, which stimulated PKC-β to invoke the IKK-IkB–NF–kB pathway to increase proliferation and S-phase fraction. The mitotic index and immunoreactivity of NF-kB, phospho-IkB, and cyclin D1 in the center of 28 large human colon, lung, and head and neck tumors exceeded that in tumor peripheries. Extracellular pressure increases $[Ca^{2+}]i$ via Cav3.3, driving a PKC-β- IKK- IkB–NF–kB pathway that stimulates cancer cell proliferation. Rapid proliferation in large stiff tumors may increase intratumoral pressure, activating this pathway to stimulate further proliferation in a feedback cycle that potentiates tumor growth. Targeting this pathway may inhibit proliferation in large unresectable tumors.

1. Introduction

Malignant tumor extracellular matrix is often stiffer than the matrix surrounding adjacent non-malignant cells (Ingber, 2008). As solid tumors expand against constraining stroma, interstitial pressure increases by 4–50 mmHg relative to pressure within normal surrounding tissues (Gutmann et al., 1992; Less et al., 1992; Raju et al., 2008). Mathematical models (Sarntinoranont et al., 2003) and direct observation suggest higher pressures within large tumors' centers decrease toward their peripheries (Boucher et al., 1990). Such increased pressure impedes perfusion and delivery of chemotherapy to

* *Corresponding author*. Department of Surgery, 4120 BPS, East Lansing, MI 48824, USA. Tel.: +1 517 884 5319; fax: +1 517 353 8957.
E-mail address: mdbasson@gmail.com (M.D. Basson).

http://dx.doi.org/10.1016/j.molonc.2014.10.008

tumors (Navalitloha et al., 2006), but the direct effects of increased extracellular pressure on the tumor cells themselves are less clear.

Prolonged pressures similar to those in tumors stimulate proliferation in mesangial cells during glomerular hypertension, in cardiac myocytes after abdominal aortic constriction, and in endothelial cells (Bevan, 1976; Kawata et al., 1998; Schwartz et al., 1999). Our preliminary study found that 15 mmHg increased pressure stimulates SW620 and HCT-116 colon cancer cell proliferation but did not define the mechanism of this effect (Walsh et al., 2004). Substrate stiffness and substrate deformation also influence cell growth in vitro (Kumar and Weaver, 2009; Paszek et al., 2005). This may occur through mechanosensitive ion channels, which influence processes ranging from bacterial turgor to growth in cardiac myocytes and epithelial cells (Hamill and Martinac, 2001).

Calcium is commonly transported by mechanosensitive ion channels and necessary for several cell processes (Hamill and Martinac, 2001). $[Ca^{2+}]i$ increases transiently in the G1/S transition of normal cells (Capiod et al., 2007) while sustained $[Ca^{2+}]i$, due to T-type channel over-expression, causes androgen-dependent LNCaP prostate cancer to assume a malignant apoptosis-resistant neuroendocrine phenotype (Mariot et al., 2002). We sought to explore whether increased extracellular pressure stimulates proliferation in cancer cells by activating a mechanosensitive calcium channel. We then further investigated calcium-sensitive mediators that modulate proliferation. This led us to the serine/threonine kinase PKC and the transcription factor NF-kB. Our preliminary work suggested that mitogenic effects of pressure in colon cancer cells require PKC and are associated with PKCα membrane translocation (Walsh et al., 2004). NF-kB modulates gene transcription in cell-cycle regulation, apoptosis, and proliferation and is activated by high pressures in the vasculature (Lemarie et al., 2003), mechanical stretch in myocytes (Kumar and Boriek, 2003), and low amplitude cyclic strain in osteoblast-like MF-63 cells (Liu et al., 2007). Furthermore, direct links between PKC and NF-kB activation have been documented in several cell lines (Sun and Yang, 2010). We hypothesized a link between extracellular pressure, calcium, and tumor proliferation.

We demonstrated that increased extracellular pressure-stimulated proliferation in 3 colon cancer, a breast cancer, and 2 prostate cancer cell lines. The SW620 colon cancer cell line was chosen as a typical model for further study, and the studies were repeated after treatment with calcium chelators and calcium-channel blockers. We identified a novel pressure-sensitive calcium channel, Cav3.3, that drives proliferation by increasing $[Ca^{2+}]i$. This Cav3.3-dependent Ca^{2+} influx promotes proliferation through PKC-β activation (not PKC-α as previously suspected), which in turn mobilizes NF-kB through the classical IKK-IkB pathway. Pressure-induced activation of these elements was Cav3.3-dependent and ultimately increased cyclin D and proliferation. To assess the clinical relevance of our findings, we compared the lower pressure peripheries to the relatively higher pressure centers of 28 large primary human tumors and demonstrated gradients in IkB phosphorylation, NF-kB, cyclin D, and proliferation in vivo consistent with the pathway delineated by our in vitro studies.

2. Materials and methods

Cells: Rat MAT-Ly-Lu-B-2 (MLL, ATCC CRL-2376), murine CT-26.CL25 (ATCC CRL-2639) and human SW620 (ATCC CCL-227), Caco-2 C2BBe1 (ATCC CRL-2102), MCF-7 (ATCC HTB-22) and PC-3 (ATCC CRL-1435) cancer cells were cultured by American Type Culture Collection (ATCC, Rockville, MD) recommendations. Cells were studied on collagen I-precoated plates.

Pressure regulation: Pressure was manipulated for 24 h utilizing an airtight box with inlet and outlet valves for gas and manometry, as previously (Downey et al., 2008).

Proliferation: MTT absorbance was assayed per ATCC protocol. Briefly, we exposed 5000 cells/well in 96 well plates to increased or ambient pressure, added MTT reagents, and quantitated 570 nm absorbance. Control and pressure-treated cells were also manually counted in 20 random fields of 6 well plates (Downey et al., 2008) with similar results.

Inhibitors: Extracellular and intracellular Ca^{2+} were chelated with 1 mM EGTA and 5 μM BAPTA-AM, respectively (EMD Chemicals, Gibbstown, NJ). 10 μM lanthanum chloride (EMD Chemicals) blocked non-specific divalent cation channels and 5 μM SKF96365 (Tocris Bioscience, Bristol, UK) blocked receptor-mediated calcium-entry (Merritt et al., 1990). Gadolinium chloride (Sigma–Aldrich, St. Louis, MO) inhibited stretch-activated ion channels (Yang and Sachs, 1989). 5 μM nimodipine [77] blocked L-type Ca^{2+} channels and 1 μM NNC 55-0396 blocked T-type channels (Tocris) (Huang et al., 2004). $NiCl_2$ (Sigma–Aldrich) blocked T-type channel subtypes, with 20 μM blocking Cav3.2 and 200 μM blocking Cav3.1 and Cav3.3 (Lee et al., 1999). 100 nM calphostin-C blocked PKC globally, 6 nM GO6976 blocked PKC-α and PKC-β, 15 nM 3-(1-(3-Imidazol-1-yl propyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione blocked PKC-β alone, and 10 nM PKC-ε translocation inhibitor peptide (EMD chemicals) blocked PKC-ε. 10 mM IKK-2 inhibitor [5-(p-fluorophenyl)-2-ureido]-thiophene-3-carboxamide, 40 nM IKK-3 inhibitor [5-(5,6-dimethoxybenzinidazol-1-yl)-3-(2-methanesulfonyl-benzyloxy)-thiophene-2-carbonitrile], and a 90 nM IKK inhibitor N(6-chloro-9H-β-carbolin-8-yl)-nicotinamide, that blocks IkB phosphorylation, were used per manufacturer's protocol separately and in combination (EMD Chemicals). 30 μM NSC23766 inhibited rac1 (EMD Chemicals). 65 nM PP2 (EMD Chemicals) was used for Src family inhibition, and Akt was inhibited using 1 μM Akt inhibitor IV (EMD Chemicals). 12 μM SN50 (EMD Chemicals) blocked NF-kB p50 nuclear localization. An SN50 inactive analog that does not affect NF-kB nuclear translocation was used as a control. 25 μM NF-kB Serine 276 inhibitory peptide (Imgenex, San Diego, CA) acts as a p65 decoy through phosphorylation at that site. Cells were treated with the inhibitory peptide or an inactive control. 1 μM TCH 021, a novel imidazoline, inhibits NF-kB gene transcription by modulating IkB degradation and subsequently inhibiting DNA binding (Kahlon et al., 2009; Peddibhotla and Tepe, 2004; Sharma et al., 2004). All inhibitors were diluted in sterile PBS, DMSO, or water and used for 24 h unless stated.

Small interfering RNA: Cav3.1, Cav3.3, PKC-β, PKC-α, and NF-kB proteins were reduced using at least siRNA specific to each protein with similar results (Cell Signaling, Beverly,