Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (3): 10-15
Published online September 12, 2014 (http://www.cellmolbiol.com)
Received on August 9, 2014, Accepted on September 5, 2014.
doi : 10.14715/cmb/2014.60.3.2

Cyclooxygenase-2 Inhibition Attenuates Hypoxic Cancer Cells Induced M2-Polarization of Macrophages

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Abstract

Tumor associated macrophages (TAMs), represent a major subpopulation of tumor infiltrating immune cells. These alternatively activated M2-polarized macrophages are well known for their pro-tumor functions. Owing to their established role in potentiating tumor-neovasculogenesis and metastasis, TAMs have emerged as promising target for anti-cancer immunotherapy. One of the key TAMs related phenomenon that is amenable to therapeutic intervention is their phenotype switching into alternatively activated M2-polarized macrophages. Hindering macrophage polarization towards a pro-tumor M2 phenotype, or better still reprogramming the M2 like TAMs towards M1 subtype is being considered a beneficial anti-cancer strategy. Hypoxic tumor milieu has been proposed as one of the most plausible factor governing M2-polarization of macrophages. We recently demonstrated that hypoxic tumor cells imparted a pro-angiogenic M2 skewed phenotype to macrophages. Furthermore, sizeable body of data indicates for participation of cyclooxygenase-2 (COX-2) in macrophage polarization. Concordantly, inhibition of COX-2 is associated with impaired macrophage polarization. Prompted by this in the current study we decided to explore if inhibition of COX-2 activity via chemical inhibitors may prevent hypoxic cancer cell induced M2-polarization of macrophages. We observed that treatment with Flunixin meglumine, an established preferential inhibitor of COX-2 activity markedly inhibited hypoxic cancer cell induced of M2-polarization of macrophages thereby indicating for usage of COX-2 inhibition as possible anti-cancer treatment modality.

Key words: M2 Polarization, COX-2, Hypoxia, Flunixin meglumine, Pro-angiogenic.

Introduction

The tumor microenvironment is comprised of a variety of non-malignant stromal cells that play a pivotal role in tumor progression and metastasis (1). These include smooth muscle cells, fibroblasts and macrophages (2). Together these stromal cells elicit instructive, permissive and inductive effects on the transformed epithelium. They act as prominent modifiers of key phenomenon involved in tumor progression such as angiogenesis and metastasis (3, 4). Macrophages are the most abundant subpopulation harboured within tumor stroma and in general are referred as tumor associated macrophages (TAMs) (5, 2). Macrophages exhibit considerable plasticity, particularly in response to microenvironmental cues by acquiring functionally distinct phenotypes (6, 7) i.e. the classically activated macrophages (M1-polarized macrophages) and alternatively activated macrophages (M2-polarized macrophages) (8, 9). While M1-polarized macrophages exhibit tumoricidal response the M2-polarized macrophages exhibit pro-tumor functions. They promote tumor neo-angiogenesis and metastasis. The TAMs express marker profile that resembles alternatively activated M2 macrophages (2). Recent studies have led to emergence of TAMs as potential targets for anticancer immunotherapy. It has been hypothesized that hindering their polarization towards a pro-tumor M2-phenotype, or better still reprogramming the M2-like TAMs towards M1-subtype may effectively counteract tumor progression.

Hypoxia i.e tumor cell oxygen deficiency is a prominent tumor micro-environmental feature. It has been proposed that macrophages initially, upon infiltrating into tumor exhibit a tumoricidal M1-polarized phenotype; however tumor micro-environmental cues subsequently polarize them into M2-skewed TAMs (10). Hypoxic tumor milieu is considered one of the most plausible stimulus evoking macrophages for phenotype switching (10). Consistent with this, the hypoxic area of human endometrial (11), breast (12, 13) prostate (14) and ovarian carcinomas (15) harbour large congregation of M2 likes TAMs. In agreement with this, we recently demonstrated that hypoxia-primed cancer cells polarize macrophages to pro-angiogenic M2-polarized subtype. Since hypoxic milieu is a persistent feature of tumor microenvironment and cannot be avoided per-se, inhibiting its consequences such as M2-polarization of macrophages could be of paramount significance for

countering tumor progression.

Epidemiological studies reveal reduced risk of cancer amongst individuals having regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, the prototypic inhibitor of COX-2 (16). Proposed mechanisms underlying anti-cancer activity of COX-2 inhibitors include diminished cell proliferation, potentiation of cancer cell apoptosis, invasion and angiogenesis, (17, 18). In addition to this a sizeable body of data indicates that COX-2 participates in macrophage polarization and inhibition of COX-2 is associated with impaired macrophage polarization. Thus anti-cancer activity of COX-2 inhibitors may also be attributed to their ability to impair M2-polarization of macrophages and thus minimize the occurrence of macrophage assisted pro-tumor phenomenon such as tumor neo-angiogenesis. Prompted by this in the current study we decided to explore if inhibition of COX-2 activity via Flunixin meglumine(2-(2-methyl-3(trifluromethyl) anilino) nicotinic acid) a well known NSAID of veterinary application, may impede hypoxic cancer cell induced M2-polarization of macrophages. In addition, attempt was made to evaluate if COX-2 inhibitors may prevent hypoxic cancer cells from imparting a pro-angiogenic function to co-cultured macrophages. The study will pave way for usage of COX-2 inhibition as possible anti-cancer treatment modality.

Materials and methods

Anti bodies and Reagents

Anti- VEGF, anti-CD206, anti-CD163 and anti-β-Actin monoclonal antibodies were purchased from Santacruz (USA). FITC conjugate of anti-CD206 antibody was procured from BD Biosciences. Alexafluor 555 conjugates of anti-mouse IgG was procured from invitrogen (USA). HRP conjugates of rabbit and mouse IgG were purchased from Cell Signaling Technology (USA) 8μm polycarbonate (PCF) cell culture inserts were purchased from Millipore (USA). Human plasma fibronectin and Phorbol 12-myristate 13-acetate (PMA) were procured from GIBCO-Invitrogen Corporation (USA) and Calbiochem (USA) respectively. Flunixin meglumine was purchased from Sigma Aldrich (USA).

Cell Culture and In Vitro Differentiation

Human leukemia monocyte THP-1 cells, human mammary cancer-derived (MDA-MB-231) cells were procured from ATCC. Cells were maintained in RPMI 1640 or DMEM respectively supplemented with 10% FBS and 100μg/ml penicillin, 0.25 μg/ml amphotericin B and 100μg/ml streptomycin in a humidified atmosphere (95% humidity) at 37°C and 5% CO_2. THP-1 cells were differentiated to macrophages according to Dockrell et al (19). The differentiation was initiated by adding 30nM phorbol 12-myristate 13-acetate (PMA) to the cells. After 3 days cells were switched to PMA free media for further 5 days so as to ensure maximal differentiation. Differentiation was ascertained by evaluating the expression of macrophage specific markers viz, CD16 and Myeloid Cell Leukemia sequence-1(Mcl-1).

Hypoxia Treatment

Cells were exposed to hypoxic environment within the hypoxia chamber (Stem cell technologies, USA) maintained at low oxygen tension (1% O_2, 5% CO_2 and 94% N_2). Treatment was initiated by introducing the cultured cells in the hypoxia chamber and replacing the existing culture medium with deoxygenated RPMI 1640/DMEM. Deoxygenated medium was prepared prior to each experiment by equilibrating the medium with a hypoxic gas mixture containing 1% O_2, 5% CO_2 and 94% N_2 at 37°C. The oxygen concentration in the hypoxic chamber and the exposure medium was monitored by using an oxygen indicator (Forma Scientific, Marietta, OH).

Fluorescence Immunocytochemistry and Flow Cytometry

The presence of M2-macrophage specific cell surface markers was detected using fluorescence immunocytochemistry and flowcytometry. For fluorescence immunocytochemical detection of M2- polarized macrophage, the culture supernatant of control and experimental macrophage cultures (grown in sterile coverslips or 8μm PCF cell culture inserts) was removed and cells were washed twice with DPBS, followed by fixation with 3.7% paraformaldehyde at 37°C. After washing with DPBS thrice, the specimen were blocked with 5% BSA for 1 hr. Thereafter cells were incubated overnight with anti-human CD206 or anti-human CD163 mouse antibodies (1:100) at 4°C. Specimens were then incubated with Alexa fluor 555 conjugated anti- mice IgG (1:100) for 1hr. Finally cells were mounted in prolong gold antifade-DAPI aqueous mounting media and visualized (200X) using Leica DCF 450C florescence microscope. For flow cytometry based detection of CD206 positive M2-macrophges, the control and experimental macrophage cultures were fixed with 3.7% paraformaldehyde for 20 min at 37°C. Thereafter cells were permealized with 0.5% TritonX for 5 minutes, washed with PBS twice and harvested for flow cytometry. Cells were suspended in PBS and incubated with FITC conjugated anti- CD206 antibody for 1 hrs at 4°C. Finally, 10,000 viable cells were analyzed using FACS Calibur flow cytometer (BD Biosciences, USA).

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (1mM phenylmethylsulfonyl fluoride, 10mg/ml aprotinin, and 10mg/ml leupeptin, 10μM sodium orthovanadate). Thereafter lysates were centrifuged at 13,000 × g at 4°C for 30 min. Cell lysate supernatants equivalent to 100 μg of protein (20) were resolved through 12% SDS-PAGE and were transferred to PVDF (Millipore, USA) membranes (21). After blocking with 5% BSA in PBS containing 0.2% Tween-20, PVDF membranes were incubated at 4°C overnight with the anti-VEGF antibody (1: 500). Blots were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:2000) and the peroxidase activity was analyzed with the ECL chemiluminiscence substrate system (USA). The expression level of various proteins was quantified by measuring the intensity of respective bands using ImageJ software (ImageJ, National Institute of Health, Bethesda, MD). Intensity of loading control i.e β-actin bands (in cell

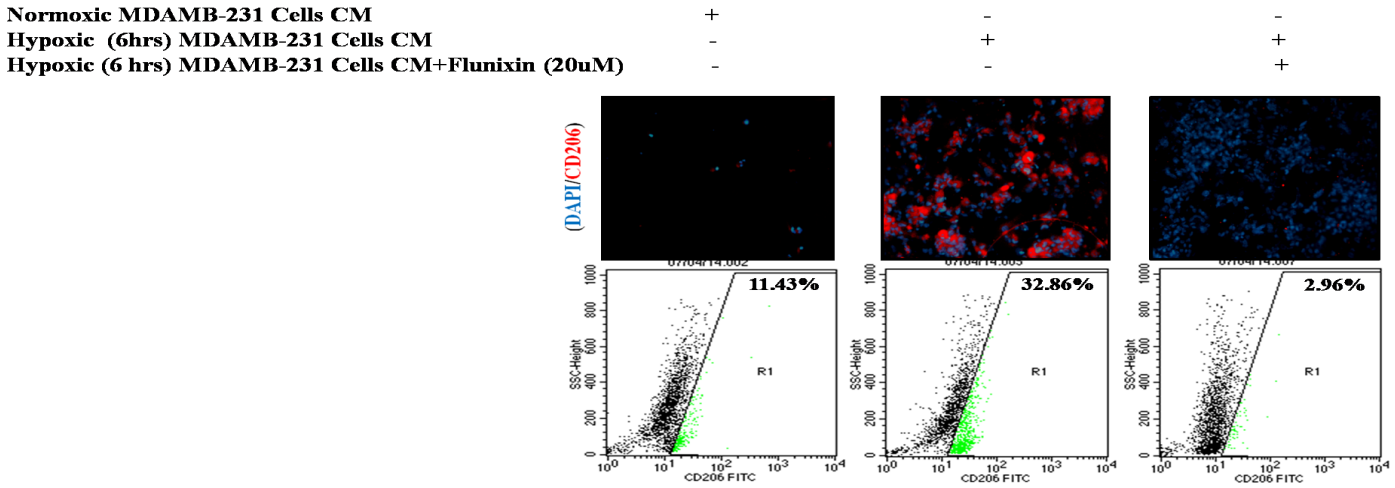

Figure 1. Flunixin meglumine Hindered Hypoxic Breast Cancers Cells Induced M2-polarization of Macrophage. THP-1 derived macrophages were incubated with conditioned media from hypoxia primed (6hrs) breast cancer cells (MDA-MB-231) CM for 24 hrs in absence or presence of COX-2 inhibitor Flunixin meglumine (20μM), followed by phenotype evaluation using immunocytochemistry and flow cytometry. Macrophages incubated with conditioned media from normoxic breast cancer cells conditioned media served as control. **(A)** Representative photomicrographs and flow cytometry data depicting enhanced M2-polarization of THP-1 derived macrophages in presence of hypoxia primed breast cancer cells conditioned media and its attenuation in presence of Flunixin meglumine as measured through immunocytochemistry and flow cytometry analysis using Alexa fluor 555 or FITC conjugated anti-CD206 antibody respectively.

lysate) ponceau's (conditioned media) was used for normalizing the expression levels.

Chick Chorioallantoic Membrane assay

To detect *in vivo* angiogenesis, we conducted Chick Chorioallantoic Membrane (CAM) assays. Approx $5x10^3$ THP-1 derived macrophages that were previously incubated with hypoxic breast cancer cells (MDAMB-231) conditioned media (CM) for 24 hrs either in absence or presence of Flunixin meglumine (20μM) were loaded on sterile gelatin sponge (4x4mm) which in turn was implanted onto the CAM at day 8 of fertilization. At day 12, CAMs were fixed with 10% formalin; the neovasculature was examined and photographed. Angiogenesis was quantified by counting the blood vessel branch points under a M205 FA Leica stereozoom microscope.

Results

Flunixin meglumine attenuates M2-polarization of THP-1 derived macrophages by hypoxia primed breast cancer cells conditioned media

We previously demonstrated that hypoxic breast cancer cells polarize macrophages towards a M2-skewed phenotype through the release of soluble mediators (21). COX-2 is a crucial enzyme associated with M2-polarization of macrophages. Thus we hypothesized that inhibiting COX-2 might hinder hypoxic breast cancer cells from inducing M2-polarization of macrophages.

To test this hypothesis we evaluated the presence of M2 specific surface marker viz. CD206 and CD163 as means to assess the extent of phenotype switching by the macrophages that were incubated with hypoxic breast cancer cells conditioned media for 24 hrs in absence or presence of Flunixin meglumine, a well known preferential COX-2 inhibitor. MDA-MB-231 human breast cancer cells were exposed to hypoxia for 6 hrs. That our experimental conditions could successfully elicit hypoxic stress in breast cancer cells was separately verified by assessing the expression profile of HIF1α, an established cellular biomarker of hypoxia. The cells maintained in normoxic conditions served as control. At the end of treatment, the hypoxic breast cancer cells conditioned media (CM) was harvested. Thereafter THP-1 derived macrophages were incubated with this conditioned media for 24 hrs in absence or presence of Flunixin meglumine (20μM). THP-1 derived macrophages incubated with normoxic breast cancer cell CM for similar duration and experimental conditions served as control. Flow cytometry and Immunocytochemistry studies revealed that macrophages grown in hypoxic breast cancer cell CM expressed M2 markers much abundantly, while the macrophages that were incubated with hypoxic breast cancer cell CM in presence of Flunixin meglumine (20μM) exhibited lesser expression of CD206 and CD163 (Fig. 1). Amongst macrophages maintained with normoxic MDA-MB231 cells CM, only 11.43 % macrophages expressed CD206 while 32.8 macrophages expressed CD206 when maintained in hypoxia (6hrs) primed breast cancer cells (MDA-MB231) CM, which showed marked decrease to 2.96% in presence of Flunixin meglumine. The results indicated for attenuation of hypoxic breast cell CM induced polarization of macrophages towards an M2 skewed phenotype (Fig.1).

Hypoxia primed bresat cancer cell induced VEGF upregulation with in macrophages was abated in presence of Flunixin meglumine.

Acquisition of pro-angiogenic phenotype is an important attribute of M2-polarized macrophages. We previously reported that hypoxic cells through soluble mediators stimulate macrophages to acquire the pro-tumor M2 polarized phenotype. Having established that Flunixin meglumine exposition was associated with impaired M2-polarization, we next decided to study the effect of Flunixin meglumine on acquisition of pro-angiogenic phenotype by macrophages. Expression level of key angiogenic mediator viz. VEGF was evaluated as an indirect measure of angiogenic potential. As expected, compared to macrophages incubated with normoxic breast cancer CM, the ones that were incubated with hy-

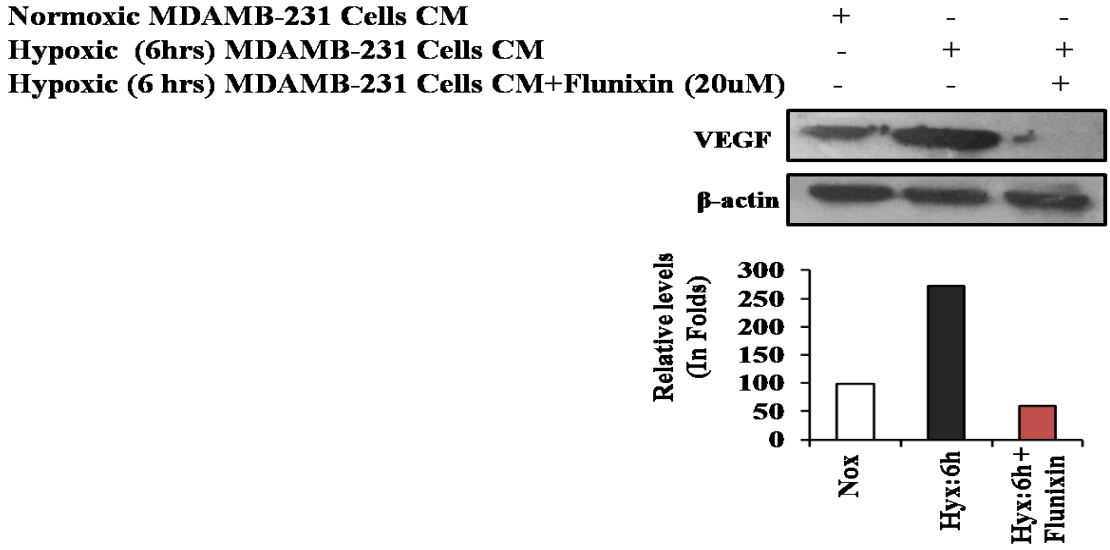

Figure 2. Flunixin meglumine Impaired Hypoxic Breast Cancers Cells Induced Potentiation of Pro-angiogenic mediators with in Macrophage (B) Representative western blot data showing hypoxia primed breast cancer cells conditioned media induced upregulation of pro-angiogenic markers viz. Vascular Endothelial Growth Factor (VEGF) with in macrophages and its downregulation following treatment with Flunixin meglumine.

poxia (6hrs) primed breast cancer CM exhibited up-regulated VEGF levels which showed marked decline in presence of Flunixin meglumine (Fig.2). Results indicated that Flunixin meglumine treatment abated hypoxia primed bresat cancer cell induced VEGF upregulation in macrophages.

Acquisition of pro-angiogenic phenotype by macrophages in presence of hypoxia primed breast cancer cells conditioned media was prevented by Flunixin meglumine.

Having established that Flunixin meglumine treatment abated hypoxia primed bresat cancer cell induced VEGF upregulation in macrophages (Fig.2), we next decided to evaluate the effect of Flunixin meglumine on angiogenic potential of macrophages. Chic chorioallantoic membrane (CAM) assay was carried out as a direct measure of angiogenic potential of macrophages exposed to different experimental conditions. Results revealed that compared to macrophages incubated with culture supernatant of normoxic breast cancer cells, the ones that were incubated with culture supernatant of hypoxic breast cancer exhibited much higher angiogenesis index which inturn showed marked reduction in presence of Flunixin meglumine (Fig.3). Results ascertained the ability of Flunixin meglumine to attenuate hypoxic cancer cell induced acquisition of pro-angiogenic phenotype by macrophages.

Discussion

Owing to rapid proliferation of cells during tumor development, hypoxia remains a persistent feature of tumor microenvironment (22, 23). Persistent hypoxia is detrimental to survival therefore in order thrive and grow in oxygen deficient environment. The rapidly proliferating dividing tumor cells must potentiate tumor neo- angiogenesis for restoring the supply of nutrients and oxygen (24). TAMs actively support hypoxic tumor cells in surviving this hostile microenvironment (25). TAMs in response to tumor microenvironmental cues

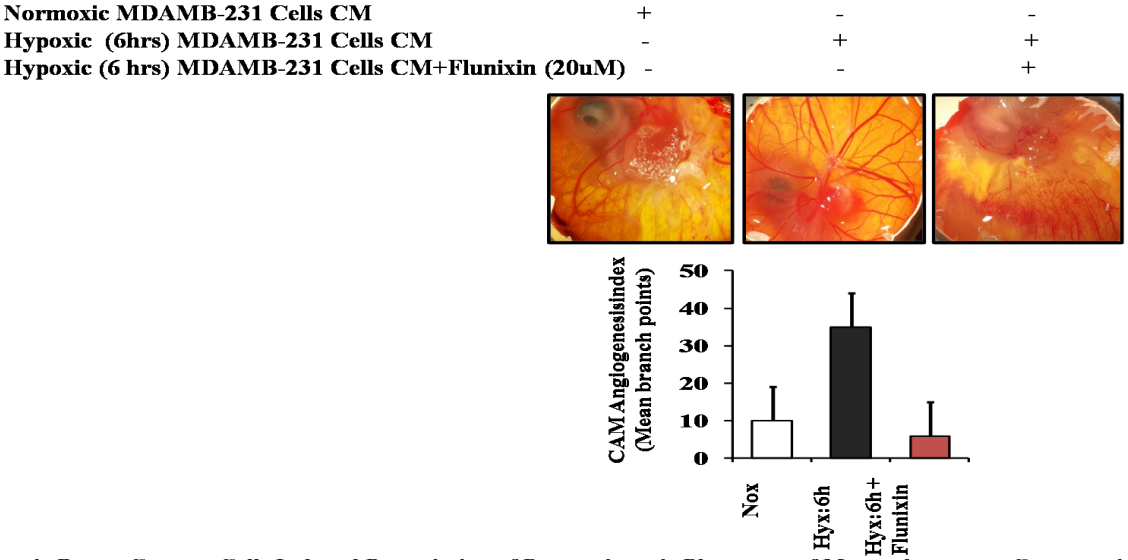

Figure 3. Hypoxic Breast Cancers Cells Induced Potentiation of Pro-angiogenic Phenotype of Macrophages was Compromised Following Flunixin meglumine Exposition: Representative CAM assay stereo-zoom micrograph and CAM angiogenesis index as a measure of angiogenic potential macrophages incubated with normoxic or hypoxic breast cancer cells CM.

undergo phenotype switching to acquire a pro-tumor M2-polarized phenotype and promote tumor progression by potentiating angiogenesis (26). Thus preventing M2-polarization of TAMs is being proposed as potential anti-cancer strategy.

COX is known to exist in two isoforms i.e. COX-1 and COX-2. While COX-1 is expressed constitutively in endothelium, kidney, stomach, COX-2 is induced specifically in response to pro-inflammatory cytokines and endotoxin (27). With regards to tumor progression, COX-2 holds larger significance as compared to COX-1. COX-2 inhibition is known to prevent breast cancer metastasis (28). COX-2 is key enzyme involved in M2 polarization of macrophages. It has been proposed that COX-2 signaling pathway is an important prerequisite for alternative activation of macrophage to M2-skewed phenotype and attenuating COX-2 activity during differentiation could markedly impede M2-polarization macrophages (28). In agreement with this selective inhibition of COX- 2 using Etodolac (1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-b]indole acetic acid) resulted in impaired macrophage M2-polarization (28). In the present study we demonstrate inhibition of macrophage M2-polarization by another COX-2 inhibitor Flunixin meglumine particularly with reference to hypoxic tumor microenvironment induced macrophage M2-polarization. We report that hypoxic breast cancer cell induced M2-polarization of THP-1 derived macrophages is markedly impaired in presence of Flunixin meglumine. Because COX-2 is associated with differentiation of monocytes into alternatively activated M2-polarized macrophages, COX-2 inhibition may impede acquisition of pro-tumor functions by TAMs. In agreement with this we observed diminished VEGF expression in

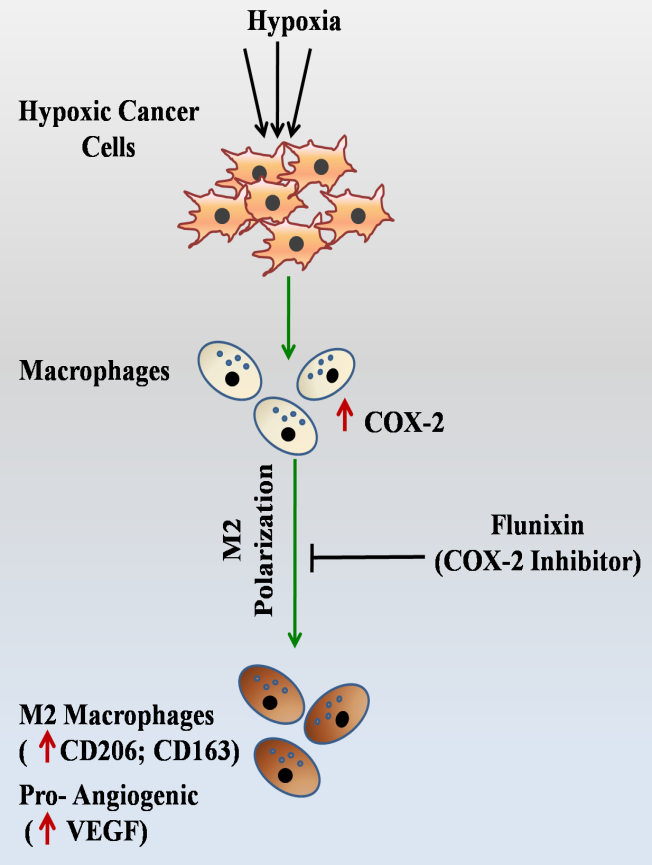

Figure 4. Schematic Representation of Flunixin meglumine inhibition of M2 polarization via COX-2 in Hypoxic Tumour area.

macrophages that were simultaneously incubated with hypoxic breast cancer cell CM and Flunixin meglumine as compared to macrophages that were incubated with hypoxic breast cancer cell CM alone. In addition these macrophages exhibited diminished angiogenic potential during in vivo CAM assay (Fig.4). Ability of Flunixin meglumine to marginally inhibit COX-1 as well may be a point of concern for usage in terms of undesirable side effects (29). Detailed experimental analysis of risk benefit ratio is an essential prerequisite before these experimental approaches could be translated to effective therapeutic regimen, nonetheless our study indicates for utility of COX-2 inhibition in devising anti-cancer modalities of improved efficacy.

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