Research Article

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Sorafenib inhibits macrophage-induced growth of hepatoma cells by interference with insulin-like growth factor-1 secretion

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Background & Aims: Hepatocellular carcinoma (HCC) associated macrophages accelerate tumor progression by growth factor release. Therefore, tumor-associated macrophages (TAM) and their initiated signaling cascades are potential therapeutic targets. Aiming at understanding anticancer effects of systemic HCC therapy, we investigated the impact of sorafenib on macrophage function, focusing on macrophage-related growth factor secretion.

Methods: Macrophage markers, cytokine and growth factor release were investigated in CSF-1 (M1) or GMCSF (M2) maturated monocyte-derived macrophages. Macrophages were treated with sorafenib (1.2–5.0 μg/ml) and culture supernatants were transferred to hepatoma cell cultures to assess growth propagation. Insulin-like growth factor (IGF) signaling was blocked with NVP-AEW541 to confirm the role of IGF-1 in macrophage-driven hepatoma cell propagation. Macrophage activation was followed by ELISA of serum soluble mCD163 in sorafenib-treated patients with HCC.

Results: Alternative macrophages (M2), which showed higher *IGF-1* ($p = 0.022$) and *CD163* mRNA ($p = 0.032$) expression compared to classical macrophages (M1), increased hepatoma growth. This effect was mediated by M2-conditioned culture media. In turn, sorafenib lowered mCD163 and IGF-1 release by M2 macrophages, which decelerated M2 macrophage driven HuH7 and HepG2 proliferation by 47% and 64%, respectively. IGF-receptor blockage with NVP-AEW541 reduced growth induction by M2-conditioned culture media in a dose dependent manner. A transient mCD163 reduction during sorafenib treatment indicated a coherent M2 macrophage inhibition in patients with HCC.

Conclusions: Sorafenib alters macrophage polarization, reduces IGF-1-driven cancer growth *in vitro* and partially inhibits macrophage activation *in vivo*. Thus macrophage modulation might contribute to the anti-cancer activity of sorafenib. However, more efficient macrophage-directed therapies are required.

Keywords: Liver cancer; Therapy; Tumor; Stroma; Immunology; Macrophages.
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Abbreviations: HCC, hepatocellular carcinoma; TAM, tumor associated macrophages; M1, classical polarized macrophages; M2, alternative polarized macrophages; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; TKI, tyrosine kinase inhibitors; CSF-1, colony-stimulating factor-1; GMCSF, granulocyte macrophage colony-stimulating factor; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IL6, interleukine-6; IL10, interleukine-10; IGBP-4, IGF binding protein-4.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death world-wide [1,2]. HCCs are associated with an estimated median survival time of 6 months if diagnosed at late clinical stages [3]. Advanced liver cirrhosis, impaired liver function, but also tumor-associated macrophages (TAM) found in the HCC environment as well as macrophage activation itself are indicators of unfavorable prognosis [4–7].

Macrophages involved in pro-inflammatory responses are categorized as classically polarized macrophages (M1), whereas those involved in tissue repair are defined as alternatively polarized macrophages (M2). TAMs typically resemble a M2-polarization within the functional spectrum of macrophages and provide tissue repair signals such as growth factors, including endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) [8,9]. Eventually growth factors released by TAM promote tumor cell proliferation and neo-angiogenesis, which support tumor progression [10].

Sorafenib is a small molecule multi-kinase inhibitor approved for systemic HCC treatment [11]. Besides interfering with tumor

Cancer

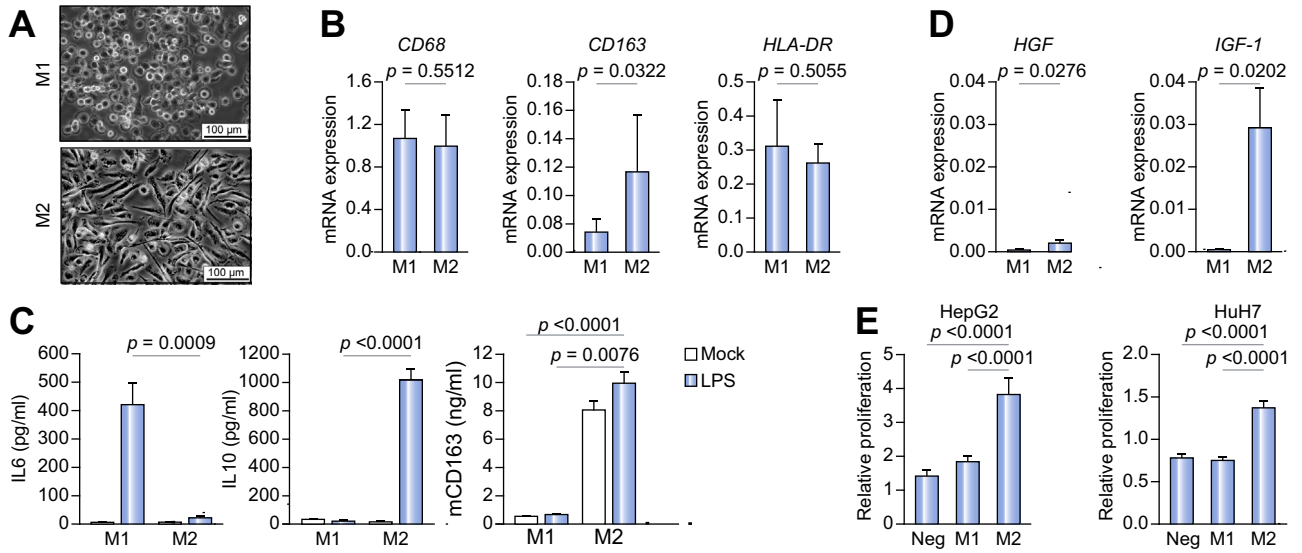

Fig. 1. Features of M1 and M2 polarized macrophages in culture. Phase contrast microscopy of M1 and M2 macrophage cultures (M1, M2) after 7 days in presence of GMCSF (800 IU/ml) or CSF-1 (100 ng/ml), respectively. (B) mRNA expression of *CD68*, *CD163*, and *HLA-DR* in M1/M2 macrophages quantified by qRT-PCR and normalized to GAPDH expression. (C) IL6, IL10, and CD163 concentrations in cell culture medium of M1/M2 macrophages after LPS stimulation for 3 h were determined by ELISA. (E) Hepatocyte growth factor (*HGF*) and insulin-like growth factor (*IGF-1*) expression in M1/M2 macrophages was determined by qRT-PCR analysis. (E) Proliferation of hepatoma cell lines (HepG2, HuH7) in the presence of M1/M2 macrophage conditioned media. Cell growth over a period of 10 days was assessed by XTT assay. Conditioned medium was added every 48 h.

growth, sorafenib has been described to perpetuate anti-cancer effector function of natural killer cells [12] and affect T cell regulation [13]. This prompted us to analyze the effect of sorafenib on macrophage-induced tumor cell growth. We investigated the impact of sorafenib on macrophage polarization as well as macrophage-dependent growth factor secretion *in vitro* and in HCC-patients, explored macrophage and tumor cell interactions during sorafenib treatment and identified IGF-1 as crucial growth factor in this context.

Material and methods

Cell preparation and culture

$CD14^+$-monocytes were isolated from PBMC by magnetic bead positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in presence of 100 ng/ml macrophage colony-stimulating factor (CSF-1) (Peprotech, Hamburg, Germany) or 800 U/ml granulocyte-macrophage colony-stimulating factor (GMCSF) (Bayer Healthcare, Leverkusen, Germany) for one week to generate M1 and M2 polarized monocyte-derived macrophages, respectively. Macrophage medium (RPMI 1640 medium, Gibco, Carlsbad, CA, USA) was supplemented with fetal calf serum (10%), L-glutamine (1%), penicillin (1%), and streptomycin (1%) (Invitrogen, Carlsbad, CA, USA). Sorafenib (sc-220125; Santa Cruz Biotech., Dallas, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) and was further diluted to 0.01% v/v in cell culture medium to reach final concentrations between 1.2 and 5 µg/µl. Mock controls refer to DMSO (0.01% v/v). After one week of differentiation sorafenib was added to the cell culture medium of monocyte-derived macrophages for 24 h, followed by a medium exchange and addition of fetal calf serum free macrophage medium for 24 h. Viability and cell amount over time was calculated via XTT assay according to the instructions provided by the supplier. Substrate turnover after standardized incubation time for 3 h was analyzed with a photometer (Infinite f200, TECAN, Männedorf, Switzerland). Serum-free macrophage medium (2 ml) was conditioned in presence of M1 or M2 macrophage cultures for 24 h. Conditioned medium was transferred on cultured HuH7 and HepG2 cells, which were seeded in a flat-bottom 96 well-plate (1000 cells/well) a night before growth kinetic assessment. Conditioned media or unconditioned control media (each 100 µl) was exchanged every 48 h and cell growth was assessed by XTT assay as outlined above. NVP-AEW541 (provided by Novartis Pharma, Basel, Switzerland) was added to the transferred media in concentrations as indicated. Macrophage cultures were stimulated with 1 ng/ml lipopolysaccharide (LPS) (Invitrogen, USA) for 3 h. Recombinant IGF-1, VEGF, and HGF were added in concentrations as indicated (all Peprotech, Germany).

Patient data and material

Following patient informed consent and local ethics committee approval at the university medical center (Mainz, Germany), tumor tissue and clinical data were obtained from patients with confirmed HCC, undergoing resection with curative intention or receiving palliative care. Clinical data were retrieved from the HCC data bank managed by the clinical registry unit (Mainz, Germany). Serum samples were obtained prior (median 1.5 months before treatment initiation), during (median 1.4 months post treatment initiation) and after (median 6.6 months post end of treatment) sorafenib administration.

Quantitative real-time PCR

Total cellular RNA extracted with Trizol® reagent (Invitrogen, USA) served as template for cDNA synthesis with Superscript III (Invitrogen, USA). Exon spanning primers specific for *IGF-1* (fw-5′-GCTGGTGGATGCTCTTCAGT-3′, rw-5′-ACTCATCCACGATGCCTGTC-3′), *HGF* (fw-5′-CGAGGCCATGGTGCTATACT-3′, rw-5′-GCATTCAGTTGTTTCCAAAGG-3′), *VEGF* (fw-5′-CCTTGCTGCTCTACCTCCAC-3′, rw-5′-GCAGTAGCTGCGCTGATAGA-3′), *GMCSF* (fw-5′-AGCAGTCAAAGGGGATGACA-3′, rw-5′-ACTACAAGCAGCACTGCCCT-3′), *CD68* (fw-5′-CGAGTCCTAGCCCAAC-3′, rw-5′-CCGCCATGTAGCTCAG-3′), *CD163* (fw-5′-TGGAGGAACAGACAAGG-3′, rw-5′-GATCCATCCAAATGCGT-3′), *HLA-DR* (fw-5′-TGTAAGGCACATGGAGGTGA-3′, rw-5′-ATAGGGCTGGAAAATGCTGA-3′) and *GAPDH* (fw-5′-ACCAACTGCTTAGCCC-3′, rw-5′-CCACGACGGACACATT-3′) were used for SYBR-green based real-time quantitative reverse-transcription PCR (qRT-PCR) in a LightCycler (Roche, Basel, Switzerland). Relative mRNA expression levels were normalized for *GAPDH*.

ELISA

Cytokine, scavenger and growth factor secretion into the macrophage culture supernatant were quantified via ELISA specific for interleukine-6 (IL6), interleukine-10 (IL10) (all BD Biosciences, Franklin Lakes, NJ, USA), CD163 (IQ-Products, Groningen, Netherlands), HGF and IGF-1 (R&D Systems, Minneapolis, MN, USA), respectively.