Biomedicine & Pharmacotherapy 67 (2013) 7–16

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Original article

Overexpression of pro-inflammatory genes and down-regulation of SOCS-1 in human PTC and in hypoxic BCPAP cells

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ARTICLE INFO

Article history:
Received 31 July 2012
Accepted 15 August 2012

Keywords:
Pro-inflammatory genes
SOCS-1
Hypoxia
Thyroid papillary carcinoma
Thyroid needle aspirate
Laser capture microdissection

ABSTRACT

Hypoxia-inducible factor-1α (HIF-1α) is frequently overexpressed and activated in many cancer types. However, its regulation and function in thyroid carcinomas are only partially known. Aim of our study was to demonstrate that adaptation to the hypoxic micro-environment by human papillary thyroid carcinoma (PTC) cells, in the absence of leukocyte infiltrate, induces a "molecular inflammation" process characterized by the expression of a large set of genes normally involved in inflammation. To address this, tumor, peritumor or normal host tissue from eleven human PTC surgical samples, were separated by laser capture microdissection (LCMD) and studied by real-time quantitative PCR and Western blot. In such condition, we observed an increased expression and activation of HIF-1α, NF-kB and pro-inflammatory genes only in tumor tissues. Importantly, an anti-inflammatory gene such as SOCS-1 was markedly down-regulated in tumor tissue compared to surrounding normal host tissue. Similar results were found in fine-needle aspiration biopsy (FNAB)-derived specimens from PTC and in hypoxic human papillary thyroid tumor cell line, BCPAP. Moreover, we also detected an elevated expression of metalloproteinase-9 (MMP9) both in solid tumor and in hypoxic-treated BCPAP cells. Our findings reveal that, in human PTC tumor, hypoxic conditions are accompanied by up-regulation of pro-inflammatory genes, down-regulation of anti-inflammatory genes and increased expression of MMP9. We propose that a better understanding of the pro- and anti-inflammatory pathways involved in the "molecular inflammation" process even in the absence of leukocyte, may help to clarify progression toward malignancy and may prove useful for new anti-tumor strategy.

1. Introduction

Papillary thyroid carcinoma (PTC) is a well-differentiated thyroid cancer derived from follicular epithelial cells and characterized by distinctive nuclear features. It represents the most common type of thyroid malignancy in humans and its incidence is increasing [1]. It is frequently associated with characteristic RET/PTC, NTRK1 and BRAF oncogenic mutations [2], which lead to activation of the common carcinogenic mitogen-activated protein kinases-(MAPK)/extracellular signal regulated kinase (ERK) pathway [3].

Notably, recent papers suggested a link between $BRAF^{V600E}$ and RET/PTC-3 oncogenes and the activation of HIF-1α and nuclear factor-kappa B (NF-kB) respectively, in papillary thyroid cancer cells [4,5]. Other authors, found an increased expression of HIF-1α in all histological type of thyroid cancer, with highest HIF-1α expression levels in the most aggressive, anaplastic thyroid carcinoma [6]. Despite a number of studies report an over-expression of HIF-1α in thyroid carcinoma, little is known about its role in promoting thyroid tumor progression, aggressiveness and metastasis.

Hypoxia represents the major stimulus for HIF-1α induction [7]. The majority of solid tumours encounter hypoxic stress as a result of both a limited oxygen diffusion due to the rapid proliferation of tumour cells and by perfusion deficits mediated by abnormal blood vessel structure within the tumour. Tumoral cells react to hypoxic conditions by triggering a survival program in the attempt to adapt to the new environment. This adaptive

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http://dx.doi.org/10.1016/j.biopha.2012.08.003

process is controlled by the transcription factor HIF-1 which rapidly reacts to low O_2 tension, activating the transcription of over one hundred genes involved in angiogenesis, glucose transport, apoptosis resistance, metastasis, inflammation, etc. [8,9].

The HIF-1 complex consists of two subunits, HIF-1α and HIF-1β Under normoxic conditions HIF-1α is degraded by the ubiquitin-proteasome system, but when the intracellular oxygen concentration drops, HIF-1α is stabilized and translocates to the nucleus where it binds to HIF-1β, activating transcription of target genes [10,11].

Another hypoxic-regulated [12,13] transcription factor is the NF-kB which plays a central role in innate immune response, inflammation and apoptosis. More recently, accumulating evidences suggest a cross-talk between the NF-kB and HIF-1α systems [14], thus linking immune response to hypoxia induced signaling and, thereafter, to cancer development and progression [12,13].

This transcription factor family contains five members named NF-kB1 (p105/p50), NF-kB2 (p100/p52), RelA (p65), RelB and c-Rel that form homo- or heterodimers which are normally held inactive in the cytosol by binding to inhibitory IkBs proteins. Upon stimulation, IkBs proteins are rapidly phosphorylated by specific IkB kinase (IKK) and degraded. Freed NF-kB dimers translocate to the nucleus where they coordinate the transcriptional activation of target genes such as anti-apoptotic genes [15], pro-inflammatory cytokines, adhesion molecules, enzymes in the prostaglandin-synthesis pathway (such as COX2), inducible nitric oxide synthase (INOS or NOS2) and proteases [16] (such as MMP9) that promote the invasive phenotype [17].

In this pathway are also involved two membrane receptors, the receptor for advanced glycation end products, Rage and the purinergic receptor, P2X7. Rage and P2X7 are both able to bind intracellular alarmins released by necrotic cells during hypoxic stress. Once expressed, both bind to their ligands and activate NF-kB pathways [18].

We have recently shown that in human breast and prostate solid tumors, as well as in breast tumor cell lines, hypoxic activation of HIF-1α and NF-kB induces a pro-inflammatory response by the tumor cells themselves which lead to the acquisition of invasive and migratory behavior [19,20]. Similarly, we observed an up-regulation of HIF-1α, followed by alarmin receptors and by NF-kB activation and overexpression of pro-inflammatory proteins in human glioblastoma multiforme (GBM) biopsies as well as in hypoxic stem cells isolated from GBM [21].

It is now accepted that chronic inflammation is the major underlying condition of a number of pathological conditions, including ageing, tumor progression, diabetes and obesity [22]. In this contest and in the presence of a leukocyte infiltrate, other groups have shown the activation of a inflammatory pathway called "molecular inflammation" [23].

Differently, in this study, we used human papillary thyroid cancer (PTC) to investigate the molecular basis of the inflammatory process, triggered by the hypoxic micro-environment and in the absence of a leukocyte infiltrate. In particular, we have analyzed a set of genes involved in inflammation and tumor progression, including those encoding membrane receptors for damage associated molecular patters (DAMPs) such as Rage and P2X7, inducible enzymes such as COX2 and NOS2, acute-phase protein such as serum amyloid protein (SAA1/2, ATN1), matrix-degrading enzymes such as MMP9 (and survival and grow factors ERα and VEGF).

We also tested our samples for an anti-inflammatory gene such as SOCS-1. SOCS family, consisting of eight members (SOCS1-7 and CIS), were originally discovered as inhibitors of cytokine signaling functioning via the Janus kinase (JAK)/signal transducers and activators of transcription (STATs) pathway. Interesting, recent data suggest that SOCS-1, but not the other members of SOCS family, regulates the duration of NF-kB signaling within the cell nucleus by decreasing p65 stability, thus exercising a negative-control (down-regulation) of the "classical" NF-kB signaling [24,25].

Our data showed that in papillary thyroid cancer (PTC), in the absence of a leukocyte infiltrate, increasing expression and activation of HIF-1α and NF-kB in malignant cells, directly correlates to one side with up-regulation of pro-inflammatory genes and to the other side with genomic and proteomic down-regulation of anti-inflammatory SOCS-1. Similar results were found in hypoxic BCPAP papillary thyroid tumor cells.

Our results suggest that the molecular inflammatory process plays a central role on malignant progression of thyroid transformed cells which can, by themselves, trigger and also modulate, a pro-inflammatory gene response leading to acquisition of a malignant phenotype.

2. Materials and methods

2.1. Surgical specimens

Thyroid tissues were obtained after thyroidectomy from patients affected by papillary thyroid carcinoma ($n = 11$; average age = 43,5 years). Each tumor, collected immediately after surgical removal, was embedded in OCT, snap-frozen in liquid nitrogen and stored at −80 °C until LCMD was performed. A representative specimen of the tumor was pathologically diagnosed. Macroscopic examination of the neoplastic nodules revealed an average size of 1.3 cm. All patients had no prior long standing anti-inflammatory treatment. Furthermore histopathology assessment of the tumor confirmed the clinical diagnosis as papillary thyroid carcinoma. Ethical approval was obtained from the Research Ethic Committees of the Azienda Policlinico Umberto I and of the Azienda Ospedaliera San Filippo Neri of Rome. All patients gave their informed consent for the study.

2.2. Fine-needle aspiration biopsy-derived (FNAB) specimens

Thyroid aspirates were collected, after signing informed consent, from patients undergoing FNAB for thyroid nodules ($n = 32$; average age 52.9 years). After cytological analysis, aspirates were classified as nodular colloid goiters ($n = 9$) or papillary thyroid carcinomas ($n = 23$). Ultrasound-guided FNAB aspirates obtained were processed as described below.

2.3. Surgical samples analysis

2.3.1. Laser capture microdissection (LCMD)

LCMD has been previously described [26]. Considerable caution was exercised to avoid removing unwanted inflammatory cells with cancer cells. All procedures are conduced under RNase-free conditions. Cell capture was completed within 1 hour to assure RNA quality. To verify the accuracy of capture, tissue sections and caps were examined post capture. The yield of total RNA was about 1 μg and the yield of total proteins was about 15 μg for each microdissected sample.

2.3.2. RNA extraction and RT-PCR

Captured cells was removed from the LCMD caps with lysis buffer (Qiagen, Hilden, Germany) and total RNA was extract using a BioRobot EZ1 workstation (Qiagen, Milan Italy). Approximately 1 μg of RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Milan, Italy) following manufacturer's instructions. Aliquots of cDNA were subjected to real-time PCR in 50 μl of 1x Universal PCR Master Mix, 0.5 μM TaqMan