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Research Article

Chronic inflammation-derived nitric oxide causes conversion of human colonic adenoma cells into adenocarcinoma cells

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ABSTRACT

It has been suggested that nitric oxide (NO) derived from chronically inflamed tissues is a cause of carcinogenesis. We herein demonstrated that administration of an inducible NO synthase inhibitor, aminoguanidine, significantly suppressed the tumorigenic conversion of human colonic adenoma (FPCK-1-1) cells into adenocarcinoma (FPCK/Inflam) cells accelerated by foreign body-induced chronic inflammation in nude mice. To determine whether NO directly promotes carcinogenesis, we exposed FPCK-1-1 cells continuously to chemically generated NO (FPCK/NO), and periodically examined their tumorigenicity. FPCK/NO cells formed tumors, whereas vehicle-treated cells (FPCK/NaOH) did not. We selected a tumorigenic population from FPCK/NO cells kept it in three-dimensional (3D) culture where in vivo-like multicellular spheroidal growth was

cAbbreviations: AG, aminoguanidine; FPCK-1-1, a human colonic adenoma cells; FPCK/Inflam, colon adenocarcinoma cells derived from FPCK-1-1 cells by chronic inflammation in vivo; FPCK/NaOH, FPCK-1-1 cells serially exposed NaOH (vehicle); FPCK/NO, FPCK-1-1 cells serially exposed to NOC18 in 2-dimensional cell culture; FPCK/NO/3D, survived cells of FPCK/NO cells after they were grown in 3D culture; NO, nitric oxide

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expected. FPCK/Inflam cells developed large spheroids whereas FPCK/NO cells formed tiny but growing compact aggregates in 3D culture. Meanwhile, FPCK-1-1 and FPCK/NaOH cells underwent anoikis (apoptotic cell death consequential on insufficient cell-to-substrate interactions) through activation of caspase 3. The survived cells in the 3D culture (FPCK/NO/3D), which were derived from FPCK/NO cells, showed a similar tumor incidence to that of FPCK/Inflam cells. These results showed that NO was one of the causative factors for the acceleration of colon carcinogenesis, especially in the conversion from adenoma to adenocarcinoma in the chronic inflammatory environment.

Introduction

Nitric oxide (NO) is a highly reactive and unstable free radical gas. It acts as a key mediator of diverse biological functions, namely, microbicidal activities, neurotransmission, vasodilatation, immune response, platelet aggregation, iron metabolism and signaling pathway [1,2]. NO is produced from L-arginine by three isoforms of NADPH-dependent NO synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [1]. The constitutive nNOS and eNOS isoforms generate NO at low concentrations (pM to nM range) for a short period (seconds/minutes) [3]. In contrast, iNOS isoform produces a large quantity of NO (μM to mM range) for extended periods (hours/days) [4,5]. The iNOS-derived NO has physiologically dual nature: the beneficial feature is antimicrobial function [1,2] and the detrimental one is involvement in pathogenesis of a variety of human diseases such as rheumatoid arthritis [6], Hashimoto's autoimmune thyroiditis [7], diabetes, systemic lupus erythematosus, septic shock, and carcinogenesis [8]. Being predominantly expressed in inflamed disorders, iNOS is closely linked with inflammation-related carcinogenesis, and chronically produced NO may account for carcinogenesis [3,9,10].

The majority of experimental and clinical studies show that iNOS and its-derived NO can induce DNA damage [11,12] or hindrance to DNA repair enzymes [3], stimulate tumor angiogenesis [3,13] or post-transcriptional modification through *S*-guanylation [14,15]; all of these potentially cause carcinogenesis [4]. Close association between NO and carcinogenesis has also been suggested from measurement of NOS activity or detection of NO-related molecules (three isoforms of NOS) or byproducts (nitrotyrosine or nitroguanine) in tumor (or pre-cancerous) tissues. However, there are few reports which demonstrate that NO could directly convert cells to tumorigenic ones. On the other hand, contradictory evidence is also reported. For instance, the $Apc^{Min/+}$ mice, which serve as a model for human familial adenomatous polyposis (FAP), develop spontaneous multiple intestinal tumors. Two study groups have reported contradictory results in the $Apc^{Min/+}$ mice crossed with *iNOS*-knockout mice: one group reported a decreased number of tumors in both small intestine and colon, suggesting that NO stimulates the intestinal tumor development [16]; the other showed a slight increase in the number of intestinal tumor in the crossed mice, implying an anti-carcinogenic function of NO [17]. Controversial results are also found in other models, a rat model for azoxymethane-induced colon carcinogenesis where an iNOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), was used. The inhibitor acted both for inhibition [18] and stimulation [19] on carcinogenesis. Likewise in the studies of human colorectal tumors, some demonstrated that iNOS was up-regulated [20], whereas others showed that iNOS was normally or down-regulated [21]. If *iNOS* gene is knocked out, other isoforms of *NOS* gene should be compensatively induced, which will add more complexity to the relationship between iNOS expression and carcinogenesis [22]. For determining whether chronic inflammation-derived NO actually causes carcinogenesis, a particular model for inflammation-based carcinogenesis and conclusive cell-based experiment is needed.

Kawaguchi et al. established an adenoma cell line (FPCK-1-1 cells) from a colonic polyp of a patient with FAP [23]. The cells were stably non-tumorigenic when 5×10^6 cells were injected subcutaneously into nude mice. However, when 1×10^5 of FPCK-1-1 cells were attached to a piece of plastic plate and implanted in a subcutaneous space formed in mice, the cells spontaneously converted themselves into progressively growing adenocarcinoma cells due to chronic inflammation induced by the foreign body, a plastic plate [24]. We previously verified that formation of highly proliferative fibrous stroma, which was induced by the foreign body implantation, was essential for the conversion of the adenoma cells [24].

In our chronic inflammation-based colon carcinogenesis model, we revealed that inflammation-derived NO accelerated colonic carcinogenesis in vivo and that NO directly converted colonic adenoma cells into adenocarcinoma cells by acquiring resistance to anoikis (apoptotic cell death due to insufficient cell-to-substrate interactions).

Materials and methods

Human colonic adenoma cells and culture condition

The origin and characteristics of the cell line used in this study have been described previously [23]. Briefly, the cell line FPCK-1-1 is one of the daughter cell lines from a FPCK-1 cell line which were established from a colonic tubular adenoma lesion developed in a male patient with familial adenomatous polyposis. Both FPCK-1 and FPCK-1-1 cell lines grow in culture, while they are non-tumorigenic in nude mice. These cell lines' phenotype is considered stable since the cells did not spontaneously acquire tumorigenicity while they were kept in culture for more than 5 years (data not shown).

FPCK-1-1 adenoma cells and its-derivative FPCKpP-4 adenocarcinoma cells (FPCK/Inflam) were maintained with a mixture of 6052 medium and DM-160 medium (Kyokutou, Tokyo, Japan) supplemented with 1% dialyzed fetal bovine serum (GIBCO BRL, Grand Island, NY), ITS premix (354350, Becton Dickinson, Tokyo, Japan), and epidermal growth factor (EGF0501, 10 ng/ml, ATGen,