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Caffeine promotes anti-tumor immune response during tumor initiation: Involvement of the adenosine A_{2A} receptor

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ABSTRACT

Epidemiologic studies depict a negative correlation between caffeine consumption and incidence of tumors in humans. The main pharmacological effects of caffeine are mediated by antagonism of the adenosine receptor, $A_{2A}R$. Here, we examine whether the targeting of $A_{2A}R$ by caffeine plays a role in anti-tumor immunity. In particular, the effects of caffeine are studied in wild-type and $A_{2A}R$ knockout ($A_{2A}R^{-/-}$) mice. Tumor induction was achieved using the carcinogen 3-methylcholanthrene (3-MCA). Alternatively, tumor cells, comprised of 3-MCA–induced transformed cells or B16 melanoma cells, were inoculated into animal footpads. Cytokine release was determined in a mixed lymphocyte tumor reaction (MLTR). According to our findings, caffeine-consuming mice (0.1% in water) developed tumors at a lower rate compared to water-consuming mice (14% *vs.* 53%, respectively, $p = 0.0286$, $n = 15$/group). Within the caffeine-consuming mice, tumor-free mice displayed signs of autoimmune alopecia and pronounced leukocyte recruitment intocarcinogen injection sites. Similarly, $A_{2A}R^{-/-}$ mice exhibited reduced rates of 3-MCA-induced tumors. In tumor inoculation studies, caffeine treatment resulted in inhibition of tumor growth and elevation in proinflammatory cytokine release over water-consuming mice, as depicted by MLTR. Addition of the adenosine receptor agonist, NECA, to MLTR resulted in a sharp decrease in IFNγ levels; this was reversed by the highly selective $A_{2A}R$ antagonist, ZM241385. Thus, immune response modulation through either caffeine or genetic deletion of $A_{2A}R$ leads to a Th1 immune profile and suppression of carcinogen-induced tumorigenesis. Taken together, our data suggest that the use of pharmacologic $A_{2A}R$ antagonists may hold therapeutic potential in diminishing the rate of cancer development.

1. Introduction

Prospective epidemiologic studies regarding the effects of caffeinated coffee on the incidence of cancer showed a negative correlation between coffee consumption and incidence of tumors in humans. In particular, meta-analysis studies comprised of >800,000 subjects revealed that caffeine consumption is associated with significantly decreased risk for cutaneous malignant melanoma [1], liver cancer [2,3] and pancreatic cancer [4]. Coffee is a leading source of methylxanthines, such as caffeine (1,3,7-trimethylxanthine), and while the immunomodulatory properties of many of the methylxanthines have been widely investigated, very few studies have directly examined the effects of caffeine on anti-tumor immune cell functions [5].

Out of the four subtypes of adenosine receptors, A_{2A} receptor ($A_{2A}R$) is the main pharmacological target of caffeine; the A_{2AR} is blocked by caffeine with a *KD* of close to 2.4 μM and 8.1 μM in humans and rats, respectively [6]. Indeed, caffeine antagonizes adenosine receptors at moderate to heavy levels of coffee consumption [7]. Adenosine, the ligand of $A_{2A}R$, is an endogenous purine nucleoside which is released from almost all cell types. Over the past few years a vast number of investigations have reported of its involvement in inflammatory processes [8]. Adenosine is constitutively present in extracellular spaces at low concentrations, but its extracellular levels dramatically increase in metabolically stressful conditions [9]. For example, adenosine accumulates

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in the hypoxic microenvironment of solid tumors [10], where its extracellular concentration is 10- to 20-fold higher than that found in adjacent normal tissue [11]. Following both its release from cells or after being derived extracellularly from related molecules, interstitial adenosine diffuses to the cell membranes of surrounding cells where it can bind to four specific G-coupled receptors, including $A_{2A}R$ [12].

Anti-tumor immune responses are coordinated by both innate immunity and adaptive immunity, and are mainly mediated by cytotoxic $CD8^+$ T cells (CTLs), natural killer (NK) cells and natural killer T (NKT) cells. In addition, dendritic cells (DCs) are important for the generation and maintenance of anti-tumor immune responses as they are able to regulate adaptive immunity and induce a CTLs response [13]. It was shown that the anti-tumor activity of these cells is strongly affected by adenosine; $A_{2A}R$ activation during T-cell stimulation significantly inhibits cytotoxicity and cytokine production [14] and has been reported to inhibit T-cell proliferation [15,16]. Similarly, using an $A_{2A}R$ agonist, CGS21680, Csoka et al. showed that $A_{2A}R$ activation suppresses the development of Th1 immune responses *in vitro* and *in vivo* [17]. In addition, T-cells that are activated in the presence of CGS21680 fail to proliferate, and then fail to produce IFNγ upon reactivation, essentially reaching T-cell anergy. Importantly, these cells maintain a suppressed phenotype after removal of the $A_{2A}R$ agonist [14,18]. In contrast, *in vivo* activation of $A_{2A}R$ was shown to drive $CD4^+$ T-cell differentiation towards Tregs, probably due to an increase in TGFβ and a decrease in IL-6 levels following $A_{2A}R$ activation [18]. In addition, $A_{2A}R$ has been shown to suppress NK cell functions, including IFNγ production [19,20]. NKT cells that are deficient in $A_{2A}R$ were found to produce significantly lower levels of IL-4, IL-10 and TGFβ, but appear to maintain IFNγ production upon stimulation with α-galactosylceramide [21].

The positive effect of caffeine and the role of $A_{2A}R$ in cancer immunity were previously demonstrated in mice inoculated with tumor cells. Ohata et al. have shown that 60% of $A_{2A}R^{-/-}$ mice completely rejected inoculated tumor cells and had survived, whereas WT mice displayed no tumor rejection and did not survive; importantly, Ohata et al. also showed that caffeine, as well as a specific $A_{2A}R$ antagonist, ZM241385, enhanced inoculated tumor cell destruction by tumor antigen-specific $CD8^+$ T cells [10]. Moreover, blockade of $A_{2A}R$ with its antagonists, SCH58261, and by caffeine, augmented the efficacy of adoptive T-cell therapy [22]. In a recent study, Beavis et al. found that CD73, which catalyzes the conversion of extracellular nucleosides to adenosine, promoted metastasis through the activation of both A_{2A} and A_{2B} adenosine receptors. $A_{2A}R^{-/-}$ mice were strongly protected against tumor metastasis whilst the blockade of $A_{2A}R$ with SCH58261 resulted in enhanced NK-cell activity *in vitro* and *in vivo* and reduced metastasis in a perforin-dependent manner [23].

The combination of A_{2A}-adenosinergic pathway inhibitors and blockade of negative immunologic regulators represents a new approach to cancer treatment. It was recently reported that combined therapy with the $A_{2A}R$ antagonist SCH58261 and PD-1 monoclonal antibody (mAb) significantly inhibited the development of B16 melanoma lung metastases and prolonged the life of 4T1.2 tumor-bearing mice compared with either monotherapy alone. Interestingly, the combination was only effective when the tumor expressed high levels of CD73 [24]. Consistent with these findings, Allard et al. showed that anti-CD73 mAb significantly enhanced the activity of both anti-CTLA-4 and anti-PD-1 mAbs against MC38-OVA and RM-1 subcutaneous tumors, and established metastatic 4T1.2 breast cancer. Furthermore, activation of $A_{2A}R$ was found to enhance PD-1 expression on tumor-specific $CD8^+$ T cells and $CD4^+$ Tregs [25].

Since evidence show that the $A_{2A}R$ pathway suppresses anti-tumor immunity, we hypothesize that part of the effects of caffeine on reducing tumor incidence may be mediated by its blockade of $A_{2A}R$. In contrast to previous studies that focused on models of tumor cell line injection, we aimed to investigate the effect of caffeine during the early stages of tumor initiation and progression in a carcinogen-induced tumor model. The role of $A_{2A}R$ in subversion of the immune response is directly examined as a possible mechanism for tumor evasion from immune-surveillance.

2. Materials and methods

2.1. Mice

ICR and C57BL/6J wild type mice were purchased from Harlan, Jerusalem, Israel; $A_{2A}R^{-/-}$ mice (ICR background) were kindly donated by Catherine Ledent (UniversitéLibre de Bruxelles) [26]. Mice were housed in the animal facility at the Soroka Medical Center (Beer-Sheva, Israel). Heterozygote $A_{2A}R^{+/-}$ mice were generated by crossbreeding with wild type ICR mice. Six- to eight-week—old ICR female mice were used in the carcinogen-induced tumor model. In the tumor cell line inoculation models, Six- to eight week-old C57BL/6J and ICR female mice were used. Animal use conformed to the guidelines established by Institutional Animal Care and Use Committee.

2.2. Adenosine and adenosine analogues

Adenosine was purchased from Sanofi Winthrop (Adenocor, Auckland, NZ). Adenosine analog 5′-(*N*-Ethylcarboxamido) adenosine (NECA) was purchased from Sigma (Rehovot, Israel). $A_{2A}R$/$A_{2B}R$ antagonist 4-(2-([7-Amino-2-(2-furyl)[1,2,4]triazin-5-ylamino) ethyl]) phenol) (ZM241385) was purchased from Tocris Cookson (Ellisville, MS, USA), and Caffeine was purchased from Sigma (cat# C0750, purity ≥ 99%).

2.3. Carcinogen-induced tumor model

Mice were injected intramuscularly into the left hip with 200 μg of the carcinogen 3-methylcholanthrene (3-MCA; cat# 213942, Sigma) or vehicle (olive oil) (total volume 200 μl) [27]. Local fibrosarcomas developed within 3–5 months. Mice were inspected twice a week for tumor development by caliper measurement. When tumors reached 1.8 cm diameter, mice were sacrificed and cell lines were generated from the removed tumor cells. For histological examination, samples were obtained 95 days post-carcinogen injection.

2.4. Caffeine treatment protocol

Mice were injected with the carcinogen 3-MCA or with tumor cell lines, and were then randomly divided into two groups; one group was allowed to drink ad-libitum water containing caffeine (0.1% wt/vol) and the other group water with no added caffeine.

2.5. Generation of MCA-induced fibrosarcoma cell lines

Tumor tissue was aseptically removed and processed for the establishment of cell lines, as previously described [27], with minor modifications. Disaggregation was performed by continuous stirring of the minced tissue for 1 h at 37 °C in the presence of 0.2% collagenase B (Boehringer Mannheim, F.R.G) in DMEM medium. The disaggregated tissue was separated from tissue debris by 40 μm BD Falcon cell strainers and was then subjected to enzymatic digestion by trypsin (10 min at 37 °C). DMEM medium and trypsin were purchased from Biological Industries (Beit Haemek, Israel). One of the established cell lines (designated MCA-12.12) was used in the current study.