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Effects of the MCP-1 synthesis inhibitor bindarit on tumorigenesis and inflammatory markers in the C3(1)/SV40Tag mouse model of breast cancer

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ABSTRACT

Breast cancer, the most deadly cancer in women, is characterized by elevated levels of inflammation within and surrounding the tumor, which can lead to accelerated growth, invasion and metastasis. Macrophages are central to the inflammatory milieu and are recruited to the tumor microenvironment by several factors including monocyte chemoattractant protein-1 (MCP-1). Using the anti-inflammatory molecule bindarit to target MCP-1, we investigated the role of this chemokine on macrophage related inflammation and mammary tumorigenesis in a transgenic mouse model of breast cancer. C3(1)/SV40Tag mice and wild type FVB/N were randomized to either control or 0.5% bindarit diet from 4 to 21 weeks of age. Tumor number and volume were recorded over time and at sacrifice. Macrophage markers as well as inflammatory meditators were examined in the tumor tissue and mammary glands. Circulating MCP-1 and IL-6 were measured by ELISA. Bindarit treatment reduced tumor number ($P < 0.05$), but did not affect tumor size, tumor weight or tumor latency in C3(1)/SV40Tag mice. Within the tumor, mRNA expression of bindarit's primary targets, MCP-1 and IL-12/p35, were significantly decreased by bindarit treatment ($P < 0.05$), and this was consistent with trends for reduced expression of TNF-α, IL-6, F4/80, CD206, and IL-10. In mammary tissue, expression of MCP-1, TNF-α, IL-6, F4/80, IL-10 and IL-12/p35 was significantly elevated in C3(1)/SV40Tag mice compared to wild type FVB/N mice, but IL-6 was the only marker decreased by bindarit treatment ($P < 0.05$). Plasma MCP-1 was highly correlated with tumor volume ($P < 0.05$); however, it was not affected by bindarit at 21 weeks of age. Similarly, circulating IL-6 was increased in C3(1)/SV40Tag mice but there was no effect of bindarit treatment. These results show that tumor multiplicity in the C3(1)/SV40Tag mouse model of breast cancer is reduced by bindarit, however these effects are independent of changes in plasma levels of MCP-1 and IL-6, but may be related to the attenuated expression of MCP-1 along with several inflammatory mediators and macrophage markers within the tumor.

1. Introduction

Breast cancer is currently the second most commonly diagnosed cancer and the leading cause of cancer related death in women in the United States [1]. Inflammation plays a significant role as a predisposing event, as well as in the promotion and progression of the disease. Macrophages are central to the inflammatory response in breast cancer; tumor associated macrophages (TAMs) contribute to tumor onset and development through the promotion of chronic inflammation, tumor cell invasion, angiogenesis and metastasis [2,3]. In breast carcinomas, TAMs can account for more than 50% of the tumor mass and high numbers of TAMs have been correlated with poor patient prognosis in 80% of tumor cases [4,5]. Further, a high macrophage density in breast carcinoma samples was reported to be a significant independent prognostic indicator of both relapse free survival and overall survival [2,5,6]. Therefore, identifying strategies to minimize the negative impact of TAMs in breast cancer is of importance.

Monocyte chemoattractant protein-1 (MCP-1) is significantly correlated with TAM accumulation in primary breast tumors [7]. In fact, MCP-1 has been suggested to be the most important chemokine for macrophage recruitment to the tumor microenvironment [8]. MCP-1 carries significant prognostic value for relapse

Abbreviations: MCP-1, monocyte chemoattractant protein-1; TAMs, tumor associated macrophages; MIN, mammary intraepithelial neoplasia; DCIS, ductal carcinoma in situ.

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free survival, is correlated with high tumor grade and lymph node metastasis, and is associated with low levels of differentiation and poor prognosis in breast cancer patients [7,9–13]. In contrast to normal breast epithelial cells which lack MCP-1 expression, high levels of MCP-1 have been observed in both invasive and non-invasive ductal carcinomas leading to the belief that MCP-1 expression may be an important aspect of tumorigenesis gained during the malignant process [14]. Accordingly, treatment of a breast cancer mouse implantation model with an MCP-1 antibody decreased macrophage number, tumor angiogenesis and volume [15]. Based on these findings MCP-1 represents a potential therapeutic target for breast cancer treatment.

Bindarit [2-((1-benzyl-indazol-3-yl)methoxy)-2-methylpropionicacid] is a well characterized small synthetic indazolic derivative best known for its transcriptional inhibition of the monocyte chemoattractant subfamily of CC chemokines including MCP-1/CCL2 [16]. Treatment of carcinomas with bindarit was first performed in a human melanoma mouse model in which twice daily injections reduced tumor volume, abolished tumor macrophage recruitment and eliminated tumor vascularization [17]. More recently, bindarit significantly decreased macrophage infiltration and local tumorigenesis in syngenic Balb/c mice injected with murine breast cancer cells [18]. However, the long-term effects of bindarit treatment in a transgenic mouse model of breast cancer have not yet been determined.

The C3(1)/SV40Tag mouse model of breast cancer exists on a FVB/N background and is representative of the human disease; lesions that develop by 8–12 weeks of age are histologically similar to mammary intraepithelial neoplasia (MIN) and ductal carcinoma in situ (DCIS) observed in humans [19,20]. Mammary tumors develop with a 100% incidence in transgenic female mice and progress to invasive carcinomas at ~16 weeks of age making this a timely and appropriate model for treatment studies [19,20]. Previously, we have shown significant elevations in plasma MCP-1 in C3(1)/SV40Tag mice compared to FVB/N control mice as well as a significant correlation between plasma MCP-1 and tumor volume [21].

The purpose of the present investigation was to characterize the role of MCP-1 on mammary tumorigenesis in the triple negative C3(1)/SV40Tag transgenic mouse model of breast cancer by using bindarit to attenuate MCP-1 expression. We hypothesized that treatment with bindarit would significantly reduce gene expression and protein concentration of MCP-1 in the tumor microenvironment and that this would be associated with a decrease in tumor macrophage expression. Furthermore, we expected that these changes would be associated with a decrease in tumorigenesis.

2. Methods

2.1. Animals

Female FVB/N mice were purchased from Harlan Sprague-Dawley Laboratories and bred with male heterozygous C3(1)/SV40Tag mice (a gift from Dr. Jeffrey Green, Chief, Transgenic Oncogenesis and Genomics Section, Laboratory of Cancer Biology and Genetics, National Cancer Institute) in the animal research facility at the University of South Carolina. Female offspring were genotyped by tail snips at 3 weeks old. Mice were maintained on a 12:12 h light-dark cycle in a low-stress environment (22 °C, 50% humidity and low noise) and provided with food and water *ad libitum*. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2. Treatment

Following weaning at 4 weeks of age, C3(1)/SV40Tag mice on an FVB/N background (n = 14–15/gr) and wild-type FVB/N mice (n = 10/gr) were randomized to either bindarit (Bin) or placebo (Con) treatment (FVB-Con, n = 10; FVB-Bin, n = 10; C3-Con, n = 15; C3-Bin, n = 14). Bindarit synthesized by Angelini (Aziende Chimiche Riunite Angelini Francesco [ACRAF], Italy) was incorporated into the AIN-76A pellet diet (BioServ, Frenchtown, NJ) at a dose of 0.5% and fed to the mice from 4 to 21 weeks of age [22,23]. This dose has been used and tested in mice and was found to achieve consistent plasma bindarit levels of ~140 μg/ml in female mice which corresponds to ~400 μM, and is within the range reported to effectively inhibit MCP-1 in vitro (Product data sheet, Angelini Research Center) [16,22,23]. Further, this level of bindarit is comparable to levels achieved after the administration of 100 mg/kg via oral gavage (personal communications with Angelini Research). Incorporation of bindarit into the diet was determined to be the preferable mode of administration as it provided the least invasive and least stressful method for consistent drug administration during this long-term experiment. While no adverse side effects have been reported with bindarit treatment, all mice were closely monitored for signs of toxicity or poor health throughout the experimental period. The control groups consumed the AIN-76A pellet diet from 4 to 21 weeks of age. Body weight as well as food and water intake were measured weekly throughout the treatment period.

2.3. Tumor progression

Beginning at 10 weeks of age, all C3(1)/SV40Tag mice were examined twice a week for palpable tumors by the same trained investigator. C3(1)/SV40Tag mice typically develop palpable mammary tumors between 12 and 16 weeks of age [19,24]. Upon palpitation of a tumor, calipers were used to measure the longest and shortest diameter of the tumor. The number of tumors within each mouse was recorded and the tumor volume was calculated for each using the formula: $0.52 \times (\text{largest diameter}) \times (\text{smallest diameter})^2$, as previously described [25].

2.4. Sacrifice and tissue collection

At 21 weeks of age all mice were sacrificed via isoflurane inhalation. Visible tumors were dissected from all ten mammary glands and measured to determine tumor weight and tumor volume as described above. All remaining thoracic mammary gland tissue was then removed from both the right and left side. This tissue was either snap frozen in liquid nitrogen for gene expression analysis or fixed in 10% neutral buffered formalin for 24 h (Fisher Scientific, Pittsburg, PA) for immunohistochemical analysis. Spleen weight was recorded as it has been positively correlated with tumorigenesis [21].

2.5. Immunohistochemistry

Mammary gland sections were processed to visualize any histopathological effects of the treatment. Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol washes. H&E staining was then performed. Images were taken using the DAKO Chromavision Systems ACIS 3 system.

2.6. Plasma MCP-1 and IL-6

At sacrifice blood was collected from the inferior vena cava. Plasma was isolated after centrifugation (10 min at 10,000g) and was then stored at −80 °C until analysis. Plasma levels of MCP-1 and IL-6 were measured using ELISA techniques (R&D Systems, Minneapolis MN) according to the manufacturer's instructions. All samples were run in duplicate when sample volume permitted.