Effect of annatto-tocotrienols supplementation on the development of mammary tumors in HER-2/neu transgenic mice

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Tocotrienols (T3), the lesser known isomers of vitamin E, have been reported to possess anticancer activity both in vitro and in vivo experimental models of rodents transplanted with parental tumors or treated with carcinogens. We investigated the effects of dietary supplementation with annatto-T3 (90% δ-T3 and 10% γ-T3) on the spontaneous development of mammary tumors in HER-2/neu transgenic mice. Underlying mechanisms of the antitumor effect were evaluated by studying apoptosis, senescent-like growth arrest, immune modulation, oxidative effect and the expression of HER-2/neu in tumoral mammary glands of transgenic mice and in vitro in human and mice tumor cell lines. Annatto-T3 supplementation delayed the development of mammary tumors, reducing the number and size of mammary tumor masses and those of lung metastases. In annatto-T3-supplemented mice, both apoptosis and senescent-like growth arrest of tumor cells were increased in mammary glands while no immune modulation was observed. In vitro, a dose-dependent inhibition of cell growth, increased apoptosis and senescent-like growth arrest and a time-dependent accumulation of reactive oxygen species were observed in tumor cells treated with annatto-T3 or purified δ-T3. Annatto-T3 reduced both HER-2/neu mRNA and p185HER-2/neu protein in tumors and in tumor cell lines. The results show that the antitumor effect of annatto-T3 supplementation in HER-2/neu transgenic mice is mainly related to the direct induction of oxidative stress, senescent-like growth arrest and apoptosis of tumor cells rather than to an immune modulation.

Introduction

Chemoprevention with natural or synthetic chemical compounds has emerged as an attractive strategy to prevent carcinogenic progression to invasive cancer. Epidemiological studies have provided evidence that high consumption of fruits and vegetables effectively decreases the risk for cardiovascular disease and cancer by improving the antioxidant capacity (1), suggesting that over two-thirds of cancers might be prevented through lifestyle modification (2).

Dietary and supplemental sources of vitamin E compounds have been demonstrated to possess unique biological properties (3) that can influence critical pathways involved in cancer (4,5), cardiovascular (6,7) and neurodegenerative disease (8). Vitamin E occurs naturally in eight isoforms of α-, β-, γ- and δ-tocopherols or tocotrienols (T3). T3, although found in low amounts in human diet, are most abundant in palm, rice and annatto. Rice contains about 50% tocopherol and 50% T3, whereas palm contains ~75% T3 and 25% tocopherol. Annatto ranks highest in T3 content (100%) and it is virtually tocopherol free.

Recent studies have identified T3 as the more effective vitamin E compounds in providing anticancer protection in comparison with the well-established tocopherols (9,10). Between health-promoting properties of T3, several lines of evidence support a beneficial effect associated with the prevention of tumor development. One of the studies investigating their role in neoplastic disorders reported that α- and γ-isomers effectively suppressed the development of sarcoma 180, Ehrlich carcinoma and invasive mammary carcinoma (11). To date, T3 have demonstrated antiproliferative activities against various cancerous cells and activation of apoptosis (12,13). We recently evaluated the effect of γ- and δ-T3 on tumor cell growth and apoptosis of human breast cancer cells. In particular, we demonstrated the efficacy of T3 in reducing the viability of human breast cancer cells through a mechanism involving both apoptotic and senescence pathways (14). However, little is known about the in vivo anticancer effects of these natural compounds of vitamin E. So far, oral administration of T3 mixture (38% γ-T3, 22% α-T3 and 12% δ-T3) in drinking water significantly suppressed spontaneous liver and lung carcinogenesis in C3H/HeN male mice (15). Furthermore, γ-T3 feeding has been reported to decrease tumor weight and to prolong the survival rate of C57BL female mice transplanted with melanoma (16). On the other hand, most data on the in vivo effect of T3 have been drawn from studies performed in young mice or rats transplanted with parental tumors or treated with carcinogens as reviewed by Viola et al. (17); although informative, these data may not be entirely relevant to the development of most cancers in humans, where the tumor in spontaneous and initiated by clonal expansion from a single cell in vivo.

Therefore, the aim of this study was to investigate the effect of dietary supplementation with T3 extracts from annatto seeds, a 10% γ- and 90% δ-T3 mixture, on the development of mammary tumors appearing spontaneously in HER-2/neu transgenic mice at early age. Underlying mechanisms of the antitumoral action were explored studying the effects of annatto-T3 on apoptosis and senescence molecular markers (p53, p21, p16 and p27), oxidative stress, HER-2/neu expression and activation, induction of senescent-like growth arrest or apoptosis in tumor cells and immunological changes.

Materials and methods

Cell culture and chemicals

Human breast adenocarcinoma SKBR3 cell line was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin (all from Invitrogen, Milan, Italy). TUBO breast cancer cell line was established in vitro from a carcinoma that spontaneously arose in BALB/c mice carrying the rat HER-2/neu proto-oncogene driven by the mouse mammary tumor virus (MMTV) promoter. TUBO cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum. The mouse T-lymphoma cells YAC-1, used in natural killer cytotoxic assay, were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Annatto-T3 oil containing 90% δ-T3 and 10% γ-T3 was obtained from DeltaGold® product (American River Nutrition, Hadley, MA). High-performance liquid chromatography (HPLC)-purified δ-T3 was purchased from Sigma–Aldrich (Milan, Italy). In in vitro experiments, breast cancer cells were treated with annatto-T3 oil dissolved in ethanol at doses ranging from 0.5 to 500 µM for the times indicated.

Animals

FVB/N HER-2/neu transgenic female mice for the activated rat neu oncogene were obtained from Charles River (Hollister, CA) and maintained under specific pathogen-free conditions under a standard 12h light/12h dark regime in our animal facilities. Mice were housed in plastic non-galvanized cages and fed with standard pellet food (Nossan, Italy) and tap water ad libitum. The study was conducted in accordance with the ethical standards and according to national and international guidelines and has been approved by the animal research ethics committee of the INRCA-IRCCS.
Experimental design

Female FVB/N mice were analyzed in three different experiments. In each experiment, 4-month-old mice were randomly divided into three groups (n = 10 for each group), which received the following doses by oral gavage for three times a week: (i) 50 mg/kg annatto-T3 in olive oil, (ii) 100 mg/kg annatto-T3 in olive oil and (iii) olive oil vehicle alone (control group). Olive oil was administered as vehicle due to its low content of vitamin E (i.e. 51 p.p.m.) (18). Once a week, all mice were palpated for the detection of mammary tumors. The neoplastic masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Tumor volume was assessed with the following formula: (length × width)²/2. Tumor multiplicity was calculated as mean number of palpable mammary carcinomas per mouse. In each group of mice, the number of lung metastases present at the end of the treatment was evaluated after staining by endotracheal infiltration with 15% china ink solution. Lungs were fixed in 4% formaldehyde and 5 ml glacial acetic acid) (19). The number of macroscopic pulmonary tumor nodules was counted in each of the five lobes. Diameter of metastatic lesions were measured with calipers and volumes were calculated by (4/3πr³), assuming the metastases were spherical (19). All assays were performed in tumor masses of 4-5 mm of diameter.

Immunophenotyping

Whole spleens were dissociated into single-cell suspensions of splenocytes using the gentleMACS Dissociator (Miltenyi Biotec, Germany). Splenocytes suspension was filtered and then stratified on Lympholyte-M (Cederlane Laboratories, Canada). After density gradient centrifugation, viable lymphocytes were washed twice with phosphate-buffered saline, counted and suspended in RPMI 1640 with 10% fetal bovine serum. The phenotype of spleen cells was analyzed using the following panel of fluorochrome isothiocyanate and phycoerythrin-labeled monoclonal antibodies: anti-CD4, anti-CD8, anti-CD49b (all from Miltenyi Biotec), anti-TCRβ, anti-CD3, anti-CD25 and anti-FOXp3 (all from eBioscience, San Diego, CA). Stained cells were analyzed by a flow cytometer Epics XL (Coulter, Miami, FL).

Natural killer assay

The cytotoxic assay was performed using a fluorimetric method as reported previously (20). YAC-1 tumor cells were labeled with carboxyfluorescein diacetate, that is a non-fluorescent, electronically neutral molecule that is cell-permeable probe, 5-(and-6)-carboxy-2',7'-dichlorofluorescein. The probe is oxidized to yield high intensity fluorescence in the presence of simple (hydrogen peroxide) and more readily taken up by intact cells. Upon oxidation, the 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, that is a non-fluorescent, electronically neutral molecule that is cell-permeable probe, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, that is a non-fluorescent, electronically neutral molecule that is cell-permeable probe, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate. The fluorescence intensity is, therefore, proportional to the amount of ROS formed.

HPLC analysis of T3 and tocopherol uptake were determined using HPLC apparatus (Varian) coupled to an electrochemical detector (EC400) with a glassy carbon working electrode operated in the oxidation mode (0.6 V versus Ag/AgCl). Briefly, cells were treated as required and then harvested. Then cells were resuspended with phosphate-buffered saline and antiproteolytics (Sigma-Aldrich) and placed on ice. An aliquot of 100 µl was used to bicinchoninic acid protein assay. After adding the internal standard tocol, 1 ml of ethanol and 3 ml of n-hexane were added in sequel and the samples were vortex mixed for 3 min after each step. The lipid fraction extracted in the hexane layer was separated by centrifugation and collected. This extraction procedure was repeated twice and the organic layer was eluted in a Pyrex tube and dried down under a stream of nitrogen at 45°C. The residue was dissolved in 200 µl of methanol and inject to the HPLC system. The results corrected for the recovery of the internal standard were expressed in microgram per milligram of protein.

Reactive oxygen species generation by flow cytometry

To monitor cellular production of reactive oxygen species (ROS), we utilized a cell-permeable probe, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, that is a non-fluorescent, electronically neutral molecule that is readily taken up by intact cells. Upon oxidation, the 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate becomes the fluorescent dye 5-(and-6)-carboxy-2',7'-dichlorofluorescein. The probe is oxidized to yield high intensity of fluorescence in the presence of simple (hydrogen peroxide) and more complex related peroxides. The 5-(and-6)-carboxy-2',7'-dichlorofluorescein fluorescence intensity is, therefore, proportional to the amount of ROS formed. Fluorescence was measured by flow cytometry using a band-pass filter centered at 530 nm. The results corrected for the recovery of the internal standard were expressed in microgram per milligram of protein.
fluorescence in energized mitochondria, whereas it is green in mitochondria with dissipated ΔΨ, suggestive of mitochondrial dysfunction. In brief, cells were treated as required, after which they were incubated for 15 min with 1 μg/ml JC-1 at 37°C. The cells were then harvested, washed with phosphate-buffered saline and analyzed by fluorescence-activated cell sorting. Since appearance of green fluorescence precedes disappearance of red fluorescence, the presence of cells with low ΔΨ was evaluated on the basis of cells exhibiting increased green fluorescence.

Statistical analysis
All experiments were performed at least three times. Differences in tumor incidence were evaluated by the Mantel–Haenszel log-rank test; differences in tumor multiplicity were evaluated by analysis of variance and Student–Newman–Keuls post hoc tests; differences in lung metastasization were evaluated by one-way analysis of variance on ranks test followed by Dunn’s test and differences in mRNA expression and in immune parameters were evaluated by Student’s t-test. Difference between means was considered significant at \( P \leq 0.05 \). Data analysis was performed with SigmaStat software version 1.03 (Jandel Scientific, Germany) and Systat 10 (SPSS) software.

Results

Effect of annatto-T3 supplementation on the kinetics of tumor growth and lung metastasization
We examined the effect of annatto-T3 on spontaneous breast carcinogenesis in HER-2/neu transgenic mice. As shown in Figure 1A, the first mammary tumor appeared in both annatto-T3-treated and untreated mice at 21 weeks of age. The tumor incidence increased progressively with increasing age of mice, affecting 50% of control mice at 22 weeks of age. In mice supplemented with annatto-T3 (100 mg/kg), the kinetics of appearance of mammary tumors was significantly delayed with control (\( P = 0.004 \)) with 50% of mice bearing tumors at week 27. The kinetics of tumor incidence in 50 mg/kg of annatto-T3-treated mice was not different from that of control animals. As shown in Figure 1B, a statistically significant difference of tumor volume was observed by 26 weeks of age in annatto-T3 (100 mg/kg)-treated mice compared with either control (\( P = 0.001 \)) or 50 mg/kg of annatto-T3-treated groups (\( P = 0.02 \)). As shown in Figure 1C, starting from 24 weeks of age, the mean of mammary tumors developing in HER-2/neu mice was significantly reduced in annatto-T3 (100 mg/kg)-supplemented group compared with control and 50 mg/kg groups (\( P < 0.05 \)). As shown in Table 1, no difference was observed on the incidence of metastasis (the mean number of metastasis was 1.0 and 0.44 for control, 50 mg/kg or 100 mg/kg of annatto-T3, respectively), but mice that received either 50 or 100 mg of annatto-T3 showed significant reduction of size of lung metastasis when compared with control animals (\( P < 0.05 \), treatments versus control). In selected experiments, we performed a 60 mg/kg supplementation with α-tocopherol acetate. No statistically significant difference between treated and control groups was observed on the kinetics of tumor incidence, on tumor volume and multiplicity (data not shown).

Biodistribution of δ-T3 in serum, liver and tumors of mice
Figure 2A shows the δ-T3 levels in normal tissues and tumors in 100 mg/kg annatto-T3 and in control groups. δ-T3 was detected in normal tissues and tumors of mice 1 h after last supplementation. The increase of δ-T3 level in serum was observed in annatto-T3 (100 mg/kg)-treated mice compared with untreated animals (171.8 ± 179.2 versus 19.4 ± 14.6 nmol/g). δ-T3 was cleared from serum within 24 h returning to control levels (13.1 ± 2.0 versus 14.5 ± 3.7 nmol/g). The same trend was observed in liver, although the difference between the levels at 1 and 24 h was not significant (80.5 ± 19.5 versus 19.1 ± 33.0 nmol/g). δ-T3 levels of <10 nmol/g were found in liver of control group mice. Surprisingly, a large amount of δ-T3 was detected in tumor masses of annatto-T3 (100 mg/kg)-treated mice, without difference between early and late time (347.1 ± 350.3 and 407.0 ± 425.6 nmol/g, respectively). δ-T3 levels of <46 nmol/g were found in tumors of control group. γ-T3 was not found in samples at the times and doses analyzed (data not shown).

Analysis of senescent-like phenotype, in situ apoptosis and HER-2/neu expression in tumor tissue from annatto-T3-supplemented mice
As shown in Figure 2B, annatto-T3 (100 mg/kg) supplementation increased the number of cells with a senescent-like phenotype in mammary tumor samples, as evidenced by the augmented number of cells with enlarged and flattened morphology and with increased SA-β-gal staining. Image analysis processing showed a significantly increased percentage area of SA-β-gal staining in tumor masses from annatto-T3-treated mice (0.529 ± 0.528) in comparison with control mice (0.158 ± 0.168; \( P = 0.002 \)). The presence of apoptosis in tumor samples obtained from untreated or annatto-T3 (100 mg/kg)-supplemented mice was then evaluated in situ using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. As shown in Figure 2C, a greater number of apoptotic cells were found in tumors from supplemented mice. The mean number of dead tumor cells in mammary gland of annatto-T3-treated mice was significantly reduced in annatto-T3 (100 mg/kg)-treated group compared with control (\( P < 0.05 \)) or 50 mg/kg of annatto-T3-treated groups (\( P = 0.02 \)). As shown in Table 1, no difference was observed on the incidence of metastasis (the mean number of metastasis was 1.0 and 0.44 for control, 50 mg/kg or 100 mg/kg of annatto-T3, respectively), but mice that received either 50 or 100 mg of annatto-T3 showed significant reduction of size of lung metastasis when compared with control animals (\( P < 0.05 \), treatments versus control). In selected experiments, we performed a 60 mg/kg supplementation with α-tocopherol acetate. No statistically significant difference between treated and control groups was observed on the kinetics of tumor incidence, on tumor volume and multiplicity (data not shown).
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or control mice was 22.2 ± 19.5 or 11.1 ± 9.6, respectively (P < 0.02). No evidence of apoptosis was present in negative control. Strong positivity was obtained in DNase-treated tumor samples (positive control).

We then analyzed the HER-2/neu protein expression and phosphorylation in tumor masses from untreated and annatto-T3 (100 mg/kg)-supplemented mice through western blot assay. As shown in Figure 2D, both expression and phosphorylation were reduced in mammary tumor from annatto-T3-treated mice compared with control mice, although the difference was not statistically significant.

No significant change of all analyzed parameters has been detected in tumor masses from 50 mg/kg-treated mice (data not shown).

Effect of annatto-T3 supplementation on immune parameters
The modulation of immune system determined by annatto-T3 supplementation was evaluated through the analysis of immune phenotype, natural killer cytotoxicity and intratumoral expression of mRNA coding for perforins and granzyme. As shown in Figure 3A, CD4⁺, CD8⁺ or TCRγδ⁺ cells or CD4⁺CD25⁺FOXP3⁺ regulatory T cells were not significantly modulated by annatto-T3 supplementation. As shown in

Fig. 2. Biodistribution of δ-T3 and effect of annatto-T3 on senescent-like phenotype, in situ apoptosis and HER-2/neu expression in tumoral mammary glands. (A) At time t = 28 weeks, after 12 weeks of oral supplementation with 100 mg/kg annatto-T3 or vehicle alone, mice were killed 1 or 24 h after last dose. The δ-T3 levels were measured in serum, liver and tumor by HPLC. Each point represents the mean ± SEM. Cryostat sections of mammary glands from control or annatto-T3-supplemented mice were examined for the presence of senescent cells (B) and apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (C) Two sections of three tumors for each mice group were analyzed. The quantization of senescent or apoptotic cells was evaluated through image analysis. (D) HER-2/neu protein has been analyzed by two independent western blot assays using anti-HER-2 and anti-pHER-2 antibodies. The graph represents mean ± SDs obtained from densitometric quantification and β-actin normalization.
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Effect of in vitro annatto-T3 on murine TUBO cell line

To establish whether the effect of in vivo supplementation with annatto-T3 was related to a direct action on tumor tissues, we analyzed the uptake, viability and intracellular ROS production in HER-2/neu+ TUBO cells. As shown in Figure 4A, annatto-T3, at concentrations ranging from 0.5 to 100 μM, inhibited the viability of TUBO cells in a dose- and time-dependent manner. Then, the level of δ-T3 in cancer cells was determined by HPLC after treatment with 50 μM of annatto-T3 for 15h. As shown in Figure 4B, a large amount of δ-T3 was internalized after 15h of incubation in TUBO cells (2.5 μg δ-T3/mg of protein extract). The levels of δ-T3 in untreated cells were undetectable.

We then investigated the generation of ROS in TUBO cells in presence of 50 μM annatto-T3. The indirect flow cytometric assay, using the probe 5-(and-6)-carboxy-2',7'-dichlorofluorescein, indicated a time-dependent ROS accumulation in treated cells (P < 0.05 versus the respective control, Figure 5C). At the same conditions, the destabilization of mitochondria was evaluated. Figure 5D shows that the treatment of TUBO cells with 50 μM annatto-T3 results in an increasing number of cells with low ΔΨ occurring in 12.7% at 4h of treatment, 77.2% within 28h and 76.8% at 44h of treatment (P < 0.05 versus the respective control).

Effect of in vitro annatto-T3 on the modulation of cell-cycle checkpoint regulators in SKBR3 tumor cells

To establish whether the effect of annatto-T3 on senescent-like growth arrest observed on tumor masses of treated mice could be related to a modulation of molecular cell-cycle checkpoint regulators, we measured p53, p21WAF1, p16INK4a and p27KIP1 in untreated and annatto-T3-treated SKBR3 cells. As shown in Figure 6A, annatto-T3 induced a significant dose-dependent upregulation of p53, p21 and p27 mRNA, as defined by a change in the extent of expression at the reverse transcription–polymerase chain reaction analysis. Although low annatto-T3 doses were sufficient to significantly increase the levels of p53 and p27 over the control, only higher dose (50 μM annatto-T3) was able to increase transcription of p21 mRNA. The increase of p21 protein has been confirmed by western blot analysis as shown in Figure 6C. No significant modulation in p16 expression was observed in analyzed samples.

Effect of in vitro annatto-T3 on HER-2/neu expression and phosphorylation

As shown in Figure 6B, the HER-2/neu mRNA expression was significantly reduced in SKBR3 cells after treatment with 25, 50 or 100 μM of annatto-T3 (0.56±0.05, 0.59±0.06 and 0.55±0.08, respectively, P

Figure 3. Effect of in vivo annatto-T3 supplementation on immune parameters. (A) Annatto-T3-supplemented mice were analyzed for phenotype of spleen leukocytes through flow cytometry. (B) Splenocytes from control and treated groups were analyzed for CD49b-positive cells (left panel) and cytotoxicity against tumor cells (right panel). Differences in immune parameters were evaluated by analysis of variance followed by Student–Newman–Keuls test when appropriate. (C) mRNA expression of granzyme and perforin in mammary adenocarcinomas of control and annatto-T3-treated mice was evaluated by PCR. Data (mean ± SD) are cumulative of three different experiments.

Figure 3B, both the number and the cytotoxicity of natural killer cells in the spleen were reduced, even if not significantly, in annatto-T3 (100 mg/ kg)-supplemented mice. Neither granzyme nor perforins were detected in tumor tissue from control or annatto-T3-supplemented mice (Figure 3C).

In vitro cytotoxicity of annatto-T3 in SKBR3 tumor cell line

The in vitro antitumor activity of annatto-T3 was first compared with HPLC-purified δ-T3 (Figure 4A). The viability of SKBR3 cells was assessed in presence of purified δ-T3 or annatto-T3 in concentrations ranging from 10 to 500 μM for 24h. As shown in Figure 4A, both compounds tested inhibited the viability of SKBR3 cells in a dose-dependent manner. Annatto-T3 was lightly less effective with respect to purified δ-T3 with IC_{50} of 36±14 and 22±7 μM. To evaluate the effect of annatto-T3 on cell proliferation, SKBR3 cells were seeded in culture flasks and cultured for 2 days, then starved overnight and, on day 3, annatto-T3 was added. As shown in Figure 4B, untreated SKBR3 cells continued their progressive growth until day 5 of culture, whereas a time- and dose-dependent inhibition of cell growth was observed in annatto-T3-treated samples. Complete growth suppression was achieved at 25 μM with cell number falling below the plating density (data not shown). To evaluate whether the inhibitory effects of annatto-T3 on SKBR3 cell viability could be due to changes in apoptotic cell death, the cells were exposed for 24h to increasing concentrations of annatto-T3 and apoptosis was measured by flow cytometry using propidium iodide staining. As shown in Figure 4C, treated SKBR3 underwent apoptosis in a dose-dependent manner, with increasing number of apoptotic cells starting from 25 μM dose and picking at 50 and 100 μM of annatto-T3 or purified δ-T3 (P at least <0.05 versus control).
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This reduction in HER-2/neu expression was associated with a dose-dependent suppression of HER-2/neu tyrosine phosphorylation, when compared with control cells (see Figure 6C and quantification in the right panel).

Discussion

In this paper, we demonstrate for the first time the anticancer activity of annatto-T3 supplementation in a spontaneous breast tumor model. The data show that (i) annatto-T3 supplementation delays the development of spontaneous mammary tumors and reduces the number and volume of tumor masses and the size of lung metastases in HER-2/neu transgenic mice; (ii) annatto-T3 supplementation induces senescent-like growth arrest and apoptosis in tumoral mammary glands; (iii) in vitro annatto-T3 induces apoptosis, senescent-like growth arrest and ROS in human and murine breast cancer cell lines; (iv) both in vivo and in vitro annatto-T3 down regulates HER-2/neu gene expression and phosphorylation.

HER-2/neu transgenic mice spontaneously develop tumors in their mammary glands at an early age (25,26). We show that oral administration with 100 mg/kg annatto-T3 before the appearance of palpable mammary tumors determines a significant slowing down of the kinetic of tumor development, a lower number and volume of tumor masses, and a larger size of lung metastasis.

Our data on the anticancer effect of annatto-T3 in HER-2/neu transgenic mice represent the first study conducted in a spontaneous breast tumor model that emulate human disease. Moreover, our data extend previous studies conducted with different T3 formulations in animal models transplanted with parental tumors or treated with carcinogens. In these studies, T3-enriched mixtures from palm oil, but not tocopherols preparation, were effective in preventing dimethylbenzanthrace-induced mammary carcinogenesis in rats (27,28). Nesaretnam et al. (29) also observed a significant delay in the onset, incidence and size of tumor growth resulting from inoculation of MCF-7 breast cancer cells in nude mice supplemented with TRF compared with the controls.

The anticancer effect of annatto-T3 was accompanied by the high δ-T3 accumulation in tumors (Figure 2A). Our data agree and expand a previous study in which high accumulation of δ-T3 in hepatocellular carcinoma HepG2 cells after in vitro δ-T3 treatment was shown to cause the death of cancer cells (15). Moreover, another study showed the accumulation of δ-T3 in pancreatic tumors after oral administration of 100 mg/kg of δ-T3 in athymic nude mice, without toxicity or side effects (30). Altogether, these findings suggest that tumor tissue is a good target for accumulation of δ-T3 and that these vitamin E isomers may reach concentrations able to block tumor growth in cancer cells.

The effectiveness of annatto-T3 treatment in reducing the size of lung metastases was already present at the lower dosage (50 mg/kg) compared with control mice (Table I). This antimitastatic effect did not significantly increase after administration of 100 mg/kg annatto-T3, revealing the lack of a dose–response relation and suggesting the possibility that the progression of metastasis may be delayed by lower annatto-T3 doses than those required to inhibit growth of primary mammary tumors. Such phenomenon could be explained through a different accumulation of bioactive vitamers in metastatic target tissues, as well as a different degree of angiogenesis and vascularization.

The effectiveness of T3 as anticancer agents has been reported previously to be related to their cytoprotective activity, to antiproliferative effects on cancer cells and to the induction of tumor cell apoptosis (12,13,17). In this study, we extend the actual knowledge on the mechanism of action of T3 providing further potential mechanisms involved in their anticancer effect. According to recent in vitro evidence on purified T3 (14), annatto-T3 supplementation was able to induce apoptosis and senescent-like terminal proliferation arrest both in vivo and in vitro. It has been suggested that senescent-like growth arrest may be a significant

![Fig. 4. Effect of in vitro annatto-T3 treatment on cell growth and apoptosis in human SKBR3 tumor cell line. (A) Dose-dependent change in cell viability was assessed using AlamarBlue assay as described in Materials and methods. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. (B) SKBR3 tumor cells were cultured in medium with or without 10, 20 and 25 μM annatto-T3 from 1 to 5 days and analyzed for cell growth. (C) SKBR3 cells were cultured in medium supplemented with 15, 20, 25, 50 and 100 μM annatto-T3 or purified δ-T3 for 24 h and analyzed for the number of apoptotic cells by propidium iodide staining. Values are means ± SD of three experiments.](image-url)
determinant of tumor response to anticancer agents in conditions in which the induction of cell death does not explain by itself the antiproliferative effect (31,32). Our data demonstrate that annatto-T3 induces senescent-like growth arrest both in vivo and in vitro. In fact, an increased number of cells positive for SA-β-gal was found in mammary tumors from annatto-T3-treated mice. Furthermore, in vitro annatto-T3 was able to slow down the kinetics of growth, to increase the number of SA-β-gal positive cells and to induce a dose-dependent upregulation of p53, p21 and p27 mRNA. Both apoptosis and senescent-like growth arrest were associated with a downregulation of HER-2/neu expression and phosphorylation in tumors from annatto-T3-supplemented mice and in tumor cell cultures. The relationship between reduced expression and activation of HER-2/neu oncogene and induction of apoptosis was reported previously by us (24) and others (33) in other experimental models. Since in transgenic mice the overexpression of HER-2/neu is under the control of the MMTV promoter, we wanted to rule out the possibility that the annatto-T3 effect on HER-2/neu was due to a modulation of MMTV promoter. The data reported in Figures 4 and 6, which have demonstrated that annatto-T3 in vitro modulates the apoptosis, the senescence markers and the HER-2/neu expression in SKBR3 tumor cells (in which HER-2/neu expression is not under the control of MMTV), confirmed that annatto-T3 does not affect the MMTV promoter.

Although vitamin E (both tocopherols and T3) is known as a potent antioxidant, the antitumor activity of vitamin E may not be associated with its antioxidant activity. Rather, we have found an increased oxidative stress in tumor cells treated with annatto-T3. The annatto-T3-dependent increased ROS may be involved in the activation of both apoptosis and senescent-like growth arrest in tumor cells. Our results agree with the pro-oxidant effect of analogs of vitamin E, which was recently described to occur in neutrophils as well as in isolated low-density lipoprotein (34,35).

In conclusion, this study demonstrates that annatto-T3 may exert important anticancer effects delaying the development and the metastasizing capacity of tumors in mice transgenic for the HER-2/neu oncogene. The antitumor effect of annatto-T3 might be related to the induction of oxidative stress, senescent-like growth arrest and
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Apoptosis in HER-2/neu overexpressing mammary tumors and in tumor cells.

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Table I. Effect of supplementation with annatto-T3 on lung metastasizing in HER-2/neu transgenic mice

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<th>Control (n = 10)</th>
<th>50 mg/kg (n = 10)</th>
<th>100 mg/kg (n = 9)</th>
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<td>Lung metastases</td>
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<td>Mean number of metastases</td>
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</tr>
<tr>
<td>Maximum size of metastases (mm³)</td>
<td>3</td>
<td>0.062</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*P ≤ 0.05 in comparison with control group.

Conflict of Interest Statement: None declared.

References


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