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Inhibition of *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis by molecular iodine (I_2) but not by iodide (I^-) treatment Evidence that I_2 prevents cancer promotion

Pablo García-Solís [a], Yunuen Alfaro [a], Brenda Anguiano [a], Guadalupe Delgado [a], Raphael C. Guzman [b], Satyabrata Nandi [b], Mauricio Díaz-Muñoz [a], Olivia Vázquez-Martínez [a], Carmen Aceves [a,*]

[a] *Instituto de Neurobiología, Universidad Nacional Autónoma de México, Km 15 Carretera Qro-SLP, Juriquilla, Querétaro 76230, Mexico*
[b] *Cancer Research Laboratory, University of California, Berkeley, CA 94720, USA*

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Abstract

We analyzed the effect of molecular iodine (I_2), potassium iodide (KI) and a subclinical concentration of thyroxine (T4) on the induction and promotion of mammary cancer induced by *N*-methyl-*N*-nitrosourea. Virgin Sprague-Dawley rats received short or continuous treatment. Continuous I_2 treated rats exhibited a strong and persistent reduction in mammary cancer incidence (30%) compared to controls (72.7%). Interruption of short or long term treatments resulted in a higher incidence in mammary cancer compared to the control groups. The protective effect of I_2 was correlated with the highest expression of the I^-/Cl^- transporter pendrin and with the lowest levels of lipoperoxidation expression in mammary glands. Triiodothyronine serum levels and Na^+/I^- symporter, lactoperoxidase, or p53 expression did not show any changes. In conclusion continuous I_2 treatment has a potent antineoplastic effect on the progression of mammary cancer and its effect may be related to a decrease in the oxidative cell environment.

Keywords: Iodine; Lipoperoxidation; Mammary cancer; MNU; Rats

1. Introduction

Reproductive history has a consistent effect on increasing or decreasing the risk of developing breast cancer. Early age at menarche, late age at menopause, and nulliparity increase the risk of a woman for developing breast cancer. Conversely, late age at menarche, early age at menopause, and early age at first pregnancy decrease this risk. However, a majority of women that develop breast cancer do not have any of these risk factors (Seidman et al., 1982). There is compelling evidence showing that endocrine systems other than the reproductive one may play a role in breast carcinogenesis. Earlier studies and several recent ones support the notion of a possible link between breast cancer and thyroid function or iodine intake (Cann et al., 2000). Although the evidence that abnormal thyroid function increases the risk of breast cancer remains controversial, subclinical hypo- or hyper-thyroidism have been associated with an increase in MNU-induced mammary cancers or with a reduction in the tumor size, respectively (Jull and Huggins, 1960; Milmore et al., 1982). The mechanisms by which thyroid hormones (TH) exert these effects are not clear. It is well established that TH alters the secretion of gonadotropins, as well as the metabolism and receptor binding of steroid hormones. Thyronines also play an important regulatory role in both growth hormone and prolactin secretion (Anderson et al., 2000). Another possible mechanism may involve iodine per se. Iodine is a well-known micronutrient essential for TH synthesis in all vertebrates, as well as a promoter of metamor-

* Corresponding author. Tel.: +52 4422381067; fax: +52 4422381038.
E-mail address: caracev@servidor.unam.mx (C. Aceves).

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phosis or transformation of life stages (pupa to larvae, larvae to adult, sessile to free life, etc) in several invertebrates. Recently several authors have proposed that iodine acts as a powerful antioxidant agent or even binds to lipids to regulate cellular proliferation (Pisarev and Gartner, 2000; Venturi et al., 2000; Smyth, 2003). Moreover, it has been well demonstrated that the gastrointestinal tract is able to uptake iodine as both molecular iodine (I_2) and iodide (I^-). The distribution of these two species in blood, stomach, skin and thyroid gland is different (Thrall and Bull, 1990). I^- appears more efficient than I_2 in restoring the thyroid gland to its normal state from the goitrous condition found in iodine deficiency. However, the opposite seems to occur in the mammary gland. Iodine deficiency has been shown to alter the structure and function of the mammary glands of rats, especially alveolar cells. I_2 is distinctly more effective than I^- in diminishing ductal hyperplasia and perilobular fibrosis in mammary glands, using the same total iodine doses in both treatments (Eskin et al., 1995).

The importance of I_2 in the treatments for mammary glands dysfunctions has been corroborated in human and animal models. Seaweeds, widely consumed in Asian countries such as *wakame*, *nori* or *mekabu* (used in sushi, soup, salads, and in powdered form as a condiment), contain high quantities of iodine in several chemical forms, i.e. iodine, I_2 and organified to proteins. They have been associated with the low incidence of benign and malignant breast disease in Japanese women (iodine average consumption in the Japanese population is 5280 μg/day versus 166–209 μg/day in Occidental populations, UK and USA, respectively) (Cann et al., 2000). Traditional Eastern breast cancer medicine has long used iodine-rich seaweeds as a cancer treatment to "soften" tumors and "reduce" nodulation. In 7,12 dimethylbenz[α]anthracene (DMBA)-induced mammary carcinoma in rats, Lugol's solution (mixture of I^- and I_2) supplementation exerts a suppressive effect on the development of mammary neoplasia (Kato et al., 1994). In subjects with iodine-deficient goiter, it is well known that Lugol's solution administration effectively reduces thyroid size. Similarly, I_2 treatment of patients with benign breast disease is accompanied by a significant bilateral reduction in breast size, in addition to causing a remission of disease symptoms, which is not observed when I^- or protein-bound iodide was administrated (Ghent et al., 1993).

Several investigators have identified and characterized two different I^- active transports, the Na^+/I^- symporter (NIS) and Cl^-/I^- transporter identified as pendrin (PEN) in several organs including thyroid and mammary gland (Carrasco, 2000; Soleimani et al., 2001; Rillema and Hill, 2003). It has been observed that sera from breast cancer patients with positive thyroid-peroxidase (TPO) antibodies exhibit a potent inhibitor effect on I^- uptake in their own breast cancer tissue as well as in positive-NIS cells cultured (Kilbane et al., 2000), however, no studies in relation to I_2 uptake are available.

In relation to a possible generation of intracellular I_2, recent data show that the mammary gland of pubertal, pregnant and lactating rats express a rapid deiodinase enzyme called type 1 (Dio1), which locally converts the prohormone T4 into the active thyroid hormone, T3. This conversion also results in high intracellular concentrations of iodine (Aceves et al., 1999). Although the chemical form of the iodine that results from deiodination has not been determined, it possibly corresponds to a different and perhaps more reactive form than I^-. This notion is supported by preliminary observations by our group showing that the $Na^{125}I$ uptake from serum exhibits a different compartmental profile than that observed for ^{125}I generated by deiodination of ^{125}I-T4 in lactating mammary gland (Aceves et al., 2005). In addition, we have found that in human breast cancer (Gallardo de la O et al., 2000), rat MNU-induced mammary cancer (Aceves et al., 2002), or in immortalized cell lines (García-Solís and Aceves, 2003), the expression of Dio1 is reduced. Deiodinase is increased in response to retinoic acid treatment only in tumors arising during the first 4–6 months or in the positive ovarian-hormone receptors human breast cancer cell line MCF-7, suggesting that cancer progression is accompanied by an impairment of iodine generation in the mammary epithelium (Aceves et al., 2002; García-Solís and Aceves, 2003).

The present experiments were designed to analyze the effect of I_2, potassium iodide (KI) and a subclinical concentration of thyroxine (T4) on the induction and promotion of mammary cancer induced by *N*-methyl-*N*-nitrosourea (MNU). We also analyzed the expression of Dio1, NIS, PEN, tumor suppressor gene p53 and the oxidative cell status present in mammary glands and tumors in the different treatments.

2. Materials and methods

2.1. Animals

Virgin, female Sprague-Dawley rats, 4 weeks of age, were obtained from the vivarium of Instituto de Neurobiología, UNAM-Juriquilla. Rats were housed in a temperature-controlled room (21 ± 1 °C) with a 12-h light/dark schedule. They were fed food (Purina rat chow; Ralston Purina Co., St. Louis, MO) and water ad libitum. All of the procedures followed UNAM and University of California Animal Care and Use Committee guidelines.

2.2. Carcinogen treatment

At 7 weeks of age, rats were anesthetized with a ketamine and xylazine (Aveco, Fort Dodge, IA) mixture (30 mg and 6 mg, respectively, per kg body weight) and treated with a single intraperitoneal injection of 50 mg/kg body weight MNU (Sigma St. Louis, MO). MNU was dissolved in 0.9% saline, pH 5.0, and heated to 50–60 °C (Thompson, 2000).

2.3. Radioiodine uptake assay

The uptake analysis of both I^- and I_2 by thyroid, mammary gland and tumors was assessed by using ^{125}I as NaI (NEN Life Science Products, Boston, MA). Oxidation of $^{125}I^-$ to $^{125}I_2$ was achieved by reacting $Na^{125}I$ with H_2O_2 and HCl according to the method described by McAlpine (1945) and Thrall et al. (1992a) in which the oxidation of I^- to I_2 is 100%. The I_2 production was corroborated by the turning of color of the solution toward red. Normal female rats and MNU-treated rats with tumors arising in the first 3 months received i.p. doses of 50 μCi/rat of either $^{125}I^-$ or $^{125}I_2$. In a parallel group of rats, 6 mg of perchlorate (ClO_4^-) was i.p. administered 2 h before ^{125}I injection. All animals were sacrificed 1 h posterior to ^{125}I administration. Thyroid, inguinal mammary glands, liver and blood were collected and their radiolabel was measured in a γ-counter (Packard, Palo Alto, CA). Only animals with similar blood radiolabel readings (>5% of differences) were included. Data were normalized as radioactivity uptake compared to liver (non-uptake organ), by the following formula: (cpm/mg for thyroid, mammary gland or tumor)/(cpm/mg for liver).

2.4. Effects of iodine treatments on mammary carcinogenesis

2.4.1. Short-term treatments with KI and I_2

At 5 weeks old, rats were sorted into three experimental groups using a randomization process and iodine treatments were started. The experimental groups were: (a) control; (b) 0.05% KI in drinking water (KI), and (c) 0.05% I_2 in drinking water (I_2). The drinking water and the water used for solutions were always distilled. 0.05% I_2 solution was made considering iodine solubility (1.33×10^{-3} M) and the concentration was corroborated by titration with sodium thiosulfate (Kenkel, 1994). After 2 weeks, MNU was administered as described above to a subgroup of rats of each experimental group. Dietary treatments were continued for 1 week after carcinogen injection. At the end of iodine treatments, four or five rats from each experimental group were sacrificed by decapitation. Mammary tissues were collected and stored frozen for RNA extraction. Remaining rats were used to evaluate mammary gland tumorigenesis. At the end of the experiment (16 weeks), animals were sacrificed and tissues were collected as described below.

2.4.2. Long-term treatments with KI, I_2 and T4

At the age of 5 weeks, rats were randomly allocated into four experimental groups: (a) control, (b) 0.05% KI in drinking water (KI); (c) 0.05% I_2 in drinking water (I_2), and (d) thyroxine in drinking water (T4, 3 μg/mL). Treatments in this experiment were started 2 weeks before MNU administration and continued for 26 weeks after carcinogen injection. Sixteen weeks after MNU injection, five rats from each experimental group were sacrificed by decapitation and blood and tissue were collected for T3 serum level and lipoperoxidation determinations, respectively.

2.5. Lipoperoxidation measurement in normal and neoplastic mammary gland

The concentration of metabolites related to lipoperoxidation were quantified in normal and neoplastic mammary gland tissue by thiobarbituric acid reaction and expressed as namoles of malondialdehyde (MDA)/mg protein (Ottolenghi, 1959). Tumor samples were obtained from control MNU-treated rats when the neoplastic tissue grew to sizes larger than 2 cm in diameter. All manipulations were made rapidly on ice to avoid peroxidation. Some modifications to the original method were introduced. To determine basal measurements a sample of homogenate (0.5–1 mg protein) was incubated for 30 min at 37 °C in a 1 mL volume 150 mM Tris buffer, pH 7.4; incubation was ended by adding 1.5 mL 20% acetic acid (adjusted to pH 3.5 with KOH) and 1.5 mL 0.8% thiobarbituric acid. Parallel samples were incubated in presence of 100 μL 50 μM $FeSO_4$ (+Fe) to increase lipid oxidation in order to quantify total lipoperoxidation. Samples were kept for 45 min in a boiling water bath, 1 mL 2% KCl was added to each sample at the end of the incubation. The colored complex formed was extracted with butanol–pyrimidin (1:1%, v/v) and detected at 532 nm. The extraction coefficient of the MDA color complex was $0.0156\,cm^{-1}\,M^{-1}$. Protein quantification was determined by the Lowry method (Hernández-Muñoz et al., 1984).

2.6. T3 circulating levels

Serum T3 levels were measured by the homologous RIA method previously standardized with intra- and interassay variation coefficients of 9% and 12.8%, respectively (Valverde-R and Aceves, 1989).

2.7. Mammary gland carcinogenesis

Rats were weighed and palpated for tumors every week beginning 1 month after carcinogen exposure during 16 or 26 weeks. A tumor was defined as a discrete palpable mass recorded for at least two consecutive weeks. Tumor incidence was calculated as the percentage of animals with one or more palpable tumors per treatment. Tumor multiplicity was calculated as the average number of tumors per animal in each treatment group. The mean latency of tumor onset for each treatment group was calculated as the mean time interval (in weeks) from MNU injection to the appearance of the first palpable tumor. When the tumors had grown to 1.5–2.0 cm in diameter, rats were anesthetized with a ketamine and xylazine mixture (30 mg/6 mg/kg body weight, respectively) and the tumors were surgically removed and processed for the different biochemical analyses. Tumor sizes were measured using calipers and the volumes were calculated by the ellipsoid formula (Thompson, 2000). Mammary tumor samples were

fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histological classification.

2.8. RT-PCR

Messenger RNAs were identified by a standardized semi-quantitative PCR procedure in which an amplicon of the structural protein cyclophilin (Cyc) was used as a control for mRNA quantity and integrity (Aceves et al., 1999). Briefly, total RNA was extracted using TRIzol reagent (Life Technologies, Inc) eluted in RNAase free water (50 μL) and stored at −70 °C. The extracted RNA (0.5–2 μg) was reverse transcribed using oligo(deoxythymidine) and a specific NIS antisense primer (Table 1). PCR was carried out in a final 50 μL volume containing: 1 μL 10 dNTP Mix (Invitrogen Life-Technologies), 5 μL 10× Buffer (KCl 500 mM, Tris–HCl pH 8.3 100 mM, gelatin 10 μg/mL), 2.5 μL 30 mM $MgCl_2$, 1 μL 10 pM oligonucleotide primers and 5 U Taq DNA polymerase and 1–2 μL cDNA. Each PCR cycle consisted of a denaturation step, 94 °C for 45 s, an annealing step—55 °C for Cyc, Dio1 and NIS, 60 °C for p53, 58 °C for PEN, 61 °C for lactoperoxidase (LPO)—during 45 s, and an extension step, 72 °C for 45 s. As controls, two different reaction mixtures were used, one containing a RT mixture without RNA and with all PCR reagents, and the second containing a sample with appropriate reactants but with water instead of cDNA. Both controls were included in every experiment. Primers used are shown in Table 1. Resultant PCR fragments were 521 bp for Cyc, 251 bp for Dio1, 377 bp for NIS; 291 bp for p53; 488 bp for PEN and 298 for LPO. Five microliters of the PCR product were electrophoresed through a 2% agarose gel containing ethidium bromide on TAE buffer. Gels were viewed under UV light, photographed, and analyzed by a computer-assisted densitometric scanning of these images. The relative abundance of different mRNAs was calculated using the values of densitometric scanning of all specific amplicons and normalized by the Cyc mRNA amplicon.

2.9. Statistical analysis

The effects of dietary treatments on mammary cancer incidence were analyzed using 2 × 2 contingency tables and a chi-square test. The effects of treatments on tumor multiplicity, tumor latency, tumor size, mRNA expression, and T3 circulating levels were analyzed using one-way ANOVA and Tukey's honest significant difference tests. Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Histological analysis

Mammary glands were evaluated both macro- and microscopically for the presence of cancer. Various combinations of papillary, cribiform or comedo mammary carcinomas were detected. No correlation between histological type of mammary cancer and treatments was observed. Fig. 1 shows representative H&E sections of normal virgin mammary gland and MNU-induced mammary carcinomas. In addition, all animals subjected to the different experimental protocols showed the same increase in body weight and normal reproductive cycles.

3.2. Radioiodine uptake

To corroborate that virgin normal and tumoral mammary glands are capable of taking up iodine, groups of animals were injected with $^{125}I^-$ or $^{125}I_2$. Fig. 2 shows that both normal and tumoral mammary glands exhibit similar label quantities independently of the chemical form of iodine injected. Also, it is observed that only the radiolabeled capture from $^{125}I^-$ injected animals was partially inhibited by perchlorate, suggesting that in $^{125}I_2$ injected animals, the mammary gland and tumors labeled capture may not depend on the NaI symporter (NIS). In contrast, thyroid gland exhibits significant differences either in the amount of labeled capture as in its perchlorate inhibition, in relation with the chemical form of iodine injection. It is evident that in $^{125}I_2$ injected animals a significantly lower labeled capture is exhibited; this capture is less sensitive to perchlorate.

3.3. Effect of short-term iodine treatments on mammary cancer induced by MNU

In this experiment we studied whether acute KI or I_2 treatments could act at the initiation step of mammary carcinogenesis induced by MNU. Table 2 shows overall cancer development in short-term (3 weeks) iodine treatments 16 weeks after MNU injection. No significant differences were

Table 1
RT-PCR primer sequences

MRNA	GenBank accession no.	Sense/antisense primer sequence (5′ → 3′)
Cyc, nt 7–526	M19533	AGA CGC CGC TGT CTC TTT TCG/CCA CAC AGT CGG AGA TGG TGA TC
Dio1, nt 377–627	X57999	GCA CCT GAC CTT CAT TTC TT/CTG GCT GCT CTG GTT CTG
NIS, nt 790–809	U60282	CCG GAT CAA CCT GAT GGA CT/CCT GAG GGT GCC ACT GTA AG
PEN, nt 1491–1978	AF167412	CAT TCT GGG GCT GGA CCT C/CCT TCG GGA CAT TCA CTT TCA
LPO, nt 1042–1339	XM_220831	AAA GCC CAG TGT GAC GAG CA/GCC GTC CAT GGT CTG AGA CT
p53, nt 287–578	X13058	CTG GCC TCT GTC ATC TTC CG/CCG TCA CCA TCA GAG CAA CG

CyC: cyclophilin; Dio1: deiodinase type I; NIS: sodium/iodide symporter; PEN: pendrin; LPO: lactoperoxidase.

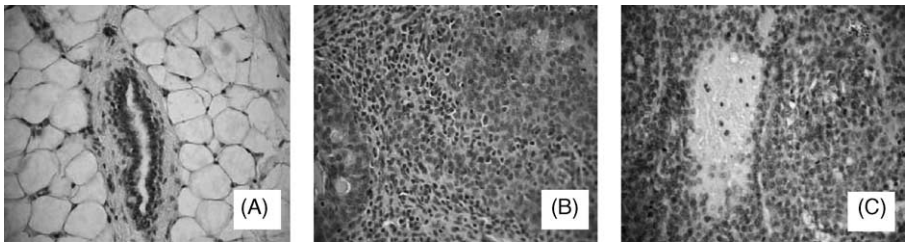

Fig. 1. H&E paraffin sections of normal and neoplastic mammary gland. (A) Mammary gland of an untreated 19-week old virgin rat, (B and C) MNU-induced mammary gland carcinomas (magnification ×40).

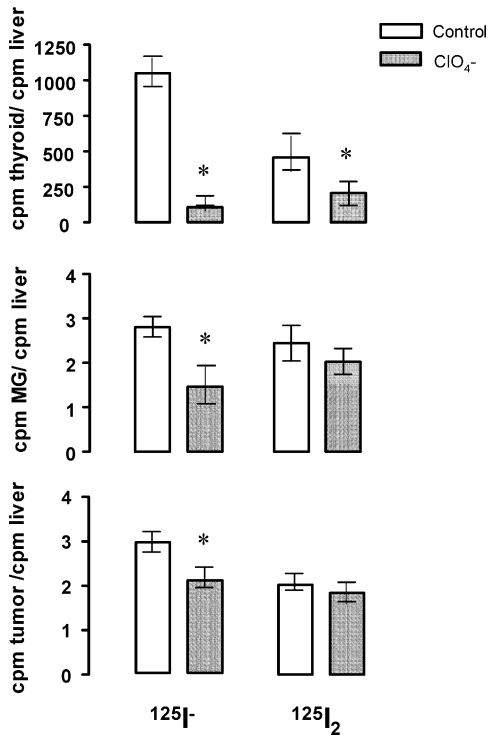

Fig. 2. $^{125}I^-$ and $^{125}I_2$ uptake in control and perchlorate (ClO_4^-) treated rats. Virgin normal and tumoral MNU-treated rats were i.p. injected with 6 mg of ClO_4^- 2 h before i.p. administration of 50 μCi of $^{125}I^-$ or $^{125}I_2$. Data were normalized as radioactivity uptake compared to liver (non-uptake organ). Values are expressed as mean ± S.D. ($n = 3$). Asterisk (*) indicates significant differences ($p < 0.05$). Abbreviation: MG, mammary gland.

found in incidence, multiplicity and tumor latency between control and iodine treatments. However, as it is shown in Fig. 3, onset of mammary carcinomas occurred earlier in those animals where I_2 treatment was discontinued. After 8 weeks post-MNU short-term I_2 treated rats presented

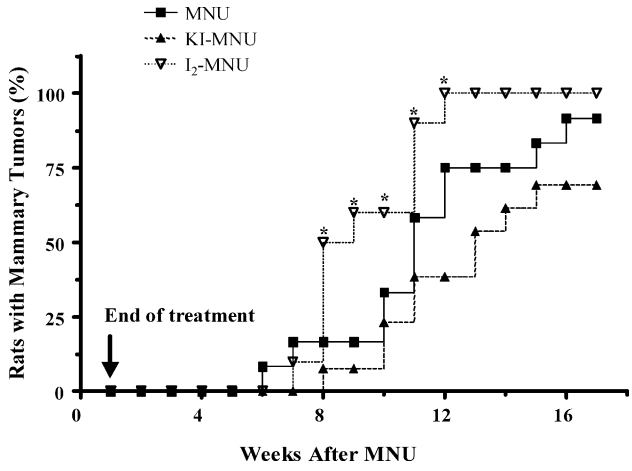

Fig. 3. Effect of short-term iodine treatments on mammary cancer incidence. Five weeks old rats were treated with 0.05% of either KI or I_2 in drinking water for 3 weeks. At 7 weeks of age MNU was administered (50 mg/kg body weight). Solid arrow indicates the end of iodine treatments. Asterisk (*) represents $p < 0.05$ compared to MNU.

50% incidence, whereas control and KI presented a less than 20% incidence. At week 12th post-MNU, short-term I_2 treated rats reached 100% mammary cancer incidence, whereas the control and KI treated animals presented only 50% and 37% incidence, respectively. These results suggest that the interruption of I_2 treatment is accompanied by an accelerated mammary tumor development.

3.4. Effect of long-term iodine and T4 treatments on mammary cancer induced by MNU

Table 3 summarizes the effect of long-term KI, I_2 and T4 treatments on mammary carcinogenesis. I_2 administration for 16 weeks after MNU was the only effective antineoplastic treatment. Fig. 4 shows the time-course of mammary cancer

Table 2
Effect of short-term KI and I_2 treatments on mammary carcinogenesis after 16 weeks of MNU administration

Treatment	No. of rats with cancer	%	Cancer latency (weeks)[a]	Carcinomas per rat[a]
MNU	11/12	91.7	11.0 ± 2.9	2.3 ± 1.4
KI-MNU	9/13	69.2	11.6 ± 2.2	1.8 ± 0.7
I_2-MNU	10/10	100	9.3 ± 1.8	1.9 ± 0.6

Five weeks old rats were treated with 0.05% of either KI or I_2 in drinking water for 3 weeks. At 7 weeks of age, rats received a single i.p. injection of MNU (50 mg/kg body weight).

[a] Mean ± S.D.

Table 3
Effect of long-term KI, I_2 and T4 treatments on mammary carcinogenesis after 16 weeks of MNU administration

Treatment	No. of rats with cancer	%	Cancer latency (weeks)[a]	Carcinomas per rat[a]
MNU	8/11	72.7	10.8 ± 0.9	1.5 ± 0.7
KI-MNU	10/11	93.7	9.8 ± 1.2	1.8 ± 0.6
I_2-MNU	3/10*	30.0*	12.7 ± 0.6	1.0 ± 0.0
T4-MNU	9/11	81.8	9.9 ± 2.9	2.5 ± 1.7

At 5 weeks of age, rats received KI (0.05%), I_2 (0.05%) or T4 (3 μg/mL) treatments in the drinking water. Two weeks later, rats received a single i.p. injection of MNU (50 mg/kg body weight) and the treatments were continued until 16 weeks elapsed.
[a] Mean ± S.D.
* Represents $p < 0.05$ compared to MNU.

incidence. On week 12th post-MNU administration, the I_2 group presented only 10% incidence of mammary cancer, whereas in both control and T4 groups the incidence was 72% and in KI 90%. On week 13th post-MNU administration, I_2 treated rats reached a 30% incidence of mammary cancer and remained that way until week 16th. The incidence in the rest of the groups rose to more than 72%. Moreover, the development of the first tumor was also delayed in I_2 treated animals compared to the other groups (12th week versus 10th week, respectively). After 16 weeks post MNU injection, the I_2 group was divided; one-half was maintained with their treatments, whereas the second half was changed to drinking water only. After 8 weeks, interrupted I_2 treatment animals reached 100% cancer incidence. This result indicates that the continuous presence of I_2 is necessary to sustain its antineoplastic effect.

3.5. Biochemical analysis

In order to establish if MNU treatments affect the uptake and/or local generation of iodine we analyzed the mRNA expression of Dio1, NIS, PEN and LPO. We also studied p53 mRNA expression to know if the protective effect of I_2 was related to an apoptotic pathway. Fig. 5 summarizes the expression of these genes in the different groups after 3 weeks of continuous treatment. Data showed that Dio1 expression is increased with I_2 treatment in normal tissue, but it is impaired in MNU treated animals. In contrast, PEN expression increased in I_2 treatment with MNU. NIS and p53 showed no changes. LPO mRNA analysis showed that this enzyme was not present in any of the groups (data not shown).

To determine if iodine treatments affected thyroid status we measured T3 circulating levels. Fig. 6 summarizes the circulating T3 levels of all groups after 16 weeks of treatment.

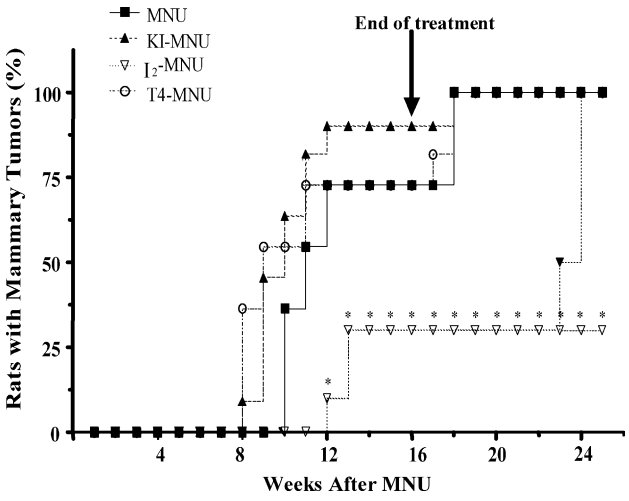

Fig. 4. Effect of long-term iodine and T4 treatments on mammary cancer incidence. Five weeks old rats received KI (0.05%), I_2 (0.05%) or T4 (3 μg/mL) treatments in the drinking water. Two weeks later animals were injected with MNU (50 mg/kg body weight), and the treatments were continued until 16 weeks. At this time, I_2 treated animals were divided; one-half maintained their treatments while the other half received only drinking water (dark inverted triangles). Asterisk (*) represents $p < 0.05$ compared to MNU.

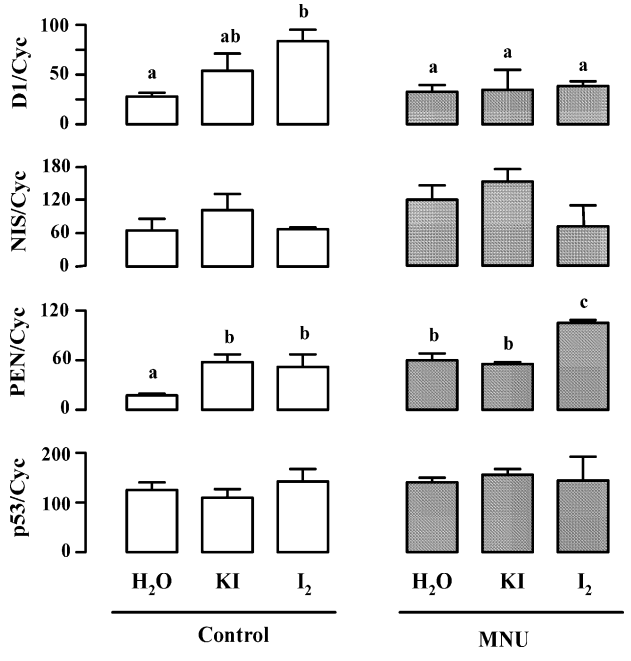

Fig. 5. Dio1, NIS, PEN and p53 mRNAs expression in mammary gland of iodine treated rats. After 3 weeks of treatment and 1 week post-MNU injection rats were sacrificed. Messenger RNA quantification was performed by semi-quantitative RT-PCR as described in Section 2. The structural protein cyclophilin (Cyc) was amplified to check for RNA quantity and integrity. The relative abundance of the different mRNAs was calculated using the values of densitometric scanning of all specific amplicons and normalized by the Cyc mRNA amplicon. Values are expressed as mean ± S.D.; the experiments were repeated three times with independent RNA samples. Different superscripts represent significant differences ($p < 0.05$).

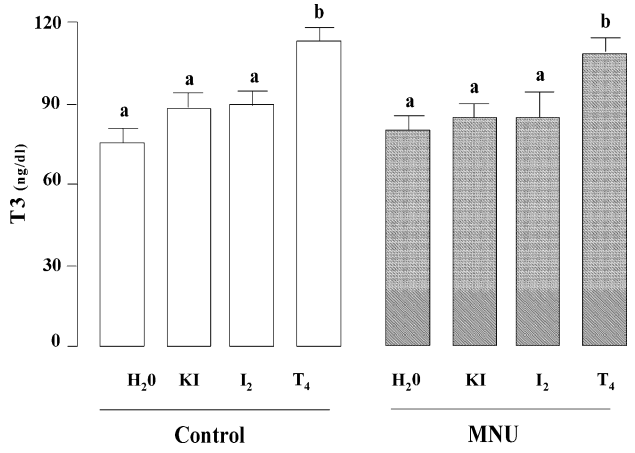

Fig. 6. Circulating T3 levels in long-term treated animals. The results represent the mean ± S.D. ($n = 5$ rats). Sixteen weeks after MNU injection rats were sacrificed. Different superscripts represent significant differences ($p < 0.05$).

Data showed that only T4 treated groups increased their circulating T3 levels.

3.6. Lipoperoxidation in normal and neoplastic mammary gland

In order to determine if iodine treatments involved an antioxidative effect, lipoperoxidation in mammary gland of rats treated with I_2 and KI with and without MNU after 16 weeks of treatment was measured. Also we include the measurement of MDA in several tumors from the control MNU-treated group which arose during the first 12 weeks after MNU-injection. Fig. 7 shows that MNU-treated glands express a higher basal lipoperoxidation than controls. It is also clear

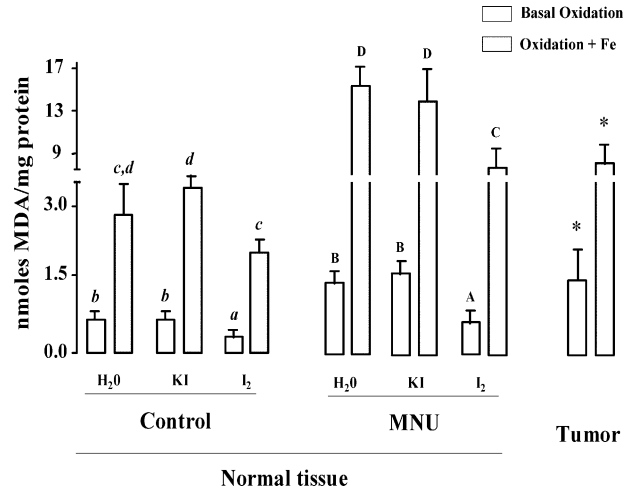

Fig. 7. Lipoperoxidation in long-term treated normal and neoplastic mammary glands. Normal and MNU treated animals were sacrificed 16 weeks after MNU injection. Tumors correspond to control animals and to those arising within the first 12 weeks after MNU injection. Values represent mean ± S.D. Different superscripts represent significant differences ($p < 0.05$). Asterisk (*) indicates significant differences when compared with H_2O control group.

that animals with I_2 treatment, with and without MNU, have a significant reduction in both basal and Fe^{2+}-induced lipoperoxidation in comparison with the other treatments. MNU-induced tumors exhibited the highest basal lipoperoxidation.

4. Discussion

This is the first report showing that I_2 is a potent protective agent against MNU-induced mammary cancer. These data agree with several other studies showing that treatments with iodine-rich seaweeds or Lugol's solution (I_2, KI mixture) have a protective effect on chemical-induced mammary carcinogenesis (Kato et al., 1994; Funahashi et al., 1999). The finding in our study that high and continuous I_2 concentrations are necessary to prevent mammary cancer incidence, indicates that its effect is achieved at the promotion level. It also corroborates epidemiological findings regarding the relative low rate of breast cancer reported in Japanese women whose normal diet is seaweed rich, as well as increasing breast cancer rates in Japanese women who immigrate or consume a Western style diet (Le Marchand et al., 1985). Besides, our data confirm that I_2 but not the I^- treatment, contribute to the maintenance of the normal integrity of mammary gland. Eskin et al. (1995) have shown that iodine deficiency alters the structure and function of rat mammary gland, especially alveolar cells, and that I_2 is distinctly more effective in diminishing ductal hyperplasia and perilobular fibrosis in the mammary glands of both rats and humans (Ghent et al., 1993; Eskin et al., 1995). Our data also show that I_2 treatment increases PEN expression suggesting a positive uptake mechanism for I_2. This proposal is reinforced by our data showing that virgin mammary glands are capable of capturing radiolabeled iodine in several forms even when the NIS has been blocked. This evidence is in agreement with previous reports showing PEN expression and the presence of the sulfate/iodide exchanger in mammary glands (Shennan, 2001; Rillema and Hill, 2003).

Another remarkable result from the present work is that MNU administration is accompanied by an impaired expression of Dio1 enzyme. This enzyme is present only during puberty, pregnancy and lactation (Aceves et al., 1995), and may represent another source of I_2 in the mammary gland. Effectively, the conversion of T4 to T3 is accompanied by the local generation of a high iodine concentration. Moreover, cancer processes in thyroid or mammary glands from animals and humans is generally accompanied by the loss of Dio1 expression (Gallardo de la O et al., 2000; Aceves et al., 2002; García-Solís and Aceves, 2003). It is possible that carcinogenetic mechanisms involve the turning off of genes related to iodine uptake or local generation.

Recently several authors have proposed that iodine acts as an antioxidant agent (Venturi et al., 2000; Smyth, 2003). In cells capable of concentrating iodine as I^-, this acts as an electron donor in the presence of H_2O_2 and peroxidases. Tseng and Latham (1984) have shown that TH reduces in

vitro lipoperoxidation of linoleic acid, even more effectively than Vitamin E, glutathione and ascorbic acid. Furthermore, it has been shown that KI is a specific scavenger of hydroxyl radicals (Murata et al., 1986), and that NaI increases more efficiently than ascorbic acid the total antioxidant status in serum of healthy donors (Winkler et al., 2000). The lack of effect evinced in the KI treatment in the present work may be explained by the absence of LPO activity in mammary glands from pubertal and virgin rats, being only present during pregnancy and lactation (Strum, 1978). LPO is a homologue protein of TPO; both enzymes are able to oxidize I^- to bind iodine to proteins or lipids. A specific iodination species has not yet been identified but several candidates exist, such as I^+ (iodinium), I^0 (iodine free radical), IO^- (hypoiodite), and I_2 (Smyth, 2003). Moreover, it has been shown that a KI excess can induce apoptosis in thyroid and cancer cells only if full TPO activity is present. In this respect, Vitale et al. (2000) show that an excess of KI induces apoptosis in thyroid cells, but if TPO activity is blocked with propylthiouracil the apoptotic effect of KI is cancelled. Besides, Zhang et al. (2003), using lung cancer cells transfected with NIS or NIS/TPO, observed that only in NIS/TPO transfected cells did a KI excess induce apoptosis.

All these data indicate that I^- from KI needs to be oxidized to have a cytotoxic effect. The possible mechanism of iodine ability to induce apoptosis is the formation of iodinated compounds such as iodolactones. The iodolactones of arachidonic acid are capable of inhibiting in vitro thyroid cell proliferation and induce apoptosis (Pisarev and Gartner, 2000; Langer et al., 2003). In the present study we did not find mRNA expression of LPO in control and MNU iodine treated rats, but in vitro experiments have shown that I_2 is able to form T4 in absence of TPO (Thrall et al., 1992b). This evidence suggests that I_2, an oxidized form of iodine, seems not to need LPO activity to be incorporated into lipids or proteins. This notion is reinforced by our findings that only the I_2 treatment was capable of diminishing basal lipoperoxidation in mammary glands. Experimental evidence reveals that free radicals like reactive oxygen species are involved in initiation and promotion of carcinogenesis, where specific mutations of certain genes like tumor suppressor gene *p53* or oncogene *ras* family occur (Ray et al., 2000). Indeed, we found in this work that the apparently "normal" cells from MNU-treated rats or the frank mammary tumors present high levels of lipoperoxidation. The exact mechanism by which I_2 prevents mammary carcinogenesis is unknown; however, it is feasible for I_2 to exhibit a dual effect. Firstly, I_2 exerts a competition with free radicals for membrane lipids and DNA to help stabilize the cells, and secondly, it induces apoptotic mechanisms through the formation of iodolactones. In this respect, we found no differences in p53 expression in any of our treatments. It is possible that I_2 treatment, besides diminishing cellular oxidative status, also participates in proliferation or apoptotic mechanisms unrelated to p53, like peroxisome proliferator-activated receptors (PPAR).

Although this study does not show a direct protective effect of I_2, our findings clearly indicate that if I_2 needs to be transformed, the resultant components are not TH or I^-. Further investigations will be necessary to elucidate the biochemical pathways involved in I_2 uptake, metabolism, and signaling.

In summary, our data show that continuous I_2 treatment is an effective inhibitor in MNU-induced mammary carcinogenesis. Its action is at the promotional level and the protective mechanisms may involve the regulation of the cell oxidative environment. Chronic I_2 treatment is not accompanied by any harmful secondary effect on the health of the animals (body weight, thyroid economy, reproductive cycle). Thus, we propose that I_2 treatment must be considered a candidate to be used in clinical trials as an adjuvant of breast cancer therapy.

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