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Immunoglobulin G from Breast Cancer Patients in Stage I Stimulates Muscarinic Acetylcholine Receptors in MCF7 Cells and Induces Proliferation. Participation of Nitric Oxide Synthase-Derived Nitric Oxide

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Abstract
Introduction Muscarinic acetylcholine receptors (mAChR) belong to the G-protein-coupled receptor family and are extensively expressed in most cells in mammals. We had reported the expression of mAChR in murine and human breast tumors.
Methods The presence of antibodies in the sera of patients with different tumors directed against self-proteins has been recently described. In this work, we investigated the presence of autoantibodies against mAChR in the sera of breast cancer patients in stage I (T1N0Mx-IgG). IgG purification was performed by affinity chromatography in protein G-agarose. We also studied the ability of these antibodies to modulate the proliferation of MCF-7 breast tumor cells by the MTS colorimetric assay. The ability of T1N0Mx-IgG to stimulate muscarinic signaling pathway via nitric oxide synthase was tested by Griess reaction.
Results We demonstrated M_3 and M_4 receptors expression in MCF-7 cells. T1N0Mx-IgG promotes cell proliferation, mimicking the action of the muscarinic agonist carbachol. This effect was preferentially due to M_3 receptor activation in tumor cells via phospholipase C-induced nitric oxide liberation by calcium-dependent nitric oxide synthases. IgG from control patients was unable to produce this effect.
Discussion IgG from patients with breast cancer in early stages could be promoting tumor progression by muscarinic activation, and its presence could be determining the prognosis of this illness.

Keywords mAChR · autoantibodies · MCF-7 breast cancer cells · proliferation · nitric oxide

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Introduction

Our concepts on the role of the immune system in the protection against cancer usually belong to two extreme attitudes. It has been suggested that immunosurveillance is responsible for detecting and eliminating tumor cells, being a central mechanism by which tumor development is kept in check. However, more recently, the cancer immunoediting hypothesis (elimination, equilibrium, and escape) predicts that in the long term, tumor infiltration by immune cells rather than protect tumor host produces great amount of soluble mediators that favor tumor progression. Moreover, this hypothesis predicts that low immune reactivity against tumors, as it is usually observed in cancer patients, is stimulating rather than inhibiting tumor cells [1]. Tumor infiltration by inflammatory cells has been detected in human and experimental tumors. Infiltrates are predominantly composed of lymphocytes and macrophages, but dendritic cells, granulocytes, and mast cells can also be present [2]. Immune cells activation was demonstrated by using antibodies (Abs) for IL-2 receptor or for class II histocompatibility antigens.

The presence of Abs in the sera of patients with different tumors has been recently described. These Abs recognize several antigens such as heat shock proteins, neuronal proteins, and nuclear proteins [3–5]. In breast cancer patients,

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B cells that infiltrate medullar mammary carcinomas respond by producing Abs against membrane autoantigens expressed on the cell surface because of tumor cell apoptosis [6].

Muscarinic acetylcholine receptors (mAChR) belong to the G-protein-coupled receptor (GPCR) superfamily and are extensively expressed in most cells in mammals. They are constituted by seven transmembrane helices. These receptors can be activated by a huge diversity of ligands including light, Ca^{2+}, odorants, peptides, proteins, etc. Five different mAChR have been identified in mammals (M_1–M_5). M_1, M_3, and M_5 subtypes are generally coupled to Gq/11 protein and activate A_2, C, or D phospholipases and/or calcium influx. M_2 and M_4 subtypes inhibit adenylyl cyclase activity by coupling to Gi protein. It has been largely documented that these receptors are extensively expressed in almost all different cells and tissues [7]. Changes in mAChR protein levels and activity have been implicated in the pathophysiology of many major infective or degenerative diseases [8, 9]. Most of the antigens identified in human tumors are self-proteins without mutations but inappropriately expressed or overexpressed. As a consequence, instead of acting as tumor-rejecting antigens, they induce tolerance or autoimmune responses to normal tissue [10, 11]. Several authors have reported overexpression of mAChR in different malignant cells [12]. We had previously reported that mAChR are spontaneously upregulated in the murine mammary adenocarcinoma cell line LM3, while they are absent in normal murine mammary cells [13]. We observed that immunoglobulin G (IgG) from early and late LM3 tumor bearers immunoblotted a protein of 70 kDa in murine tumor cells and in heart homogenates that was also recognized by a specific anti-M_2 receptor monoclonal antibody. We also observed that IgG purified from early tumor bearers stimulated LM3 cells proliferation in a more effective manner than the muscarinic agonist carbachol (CARB) did. IgG from late murine tumor bearers potentiated LM3 cells-induced angiogenesis, by increasing blood vessels number and vascular endothelial growth factor A production in peritumoral skin "via" mAchR activation, in an agonist similar manner [14]. In this work, we investigate the presence of auto-Abs against mAChR in the sera of breast cancer patients in stage I. We also studied the ability of these Abs to modulate MCF-7 cells proliferation through muscarinic signaling via phospholipase C (PLC)-nitric oxide synthase (NOS) derived nitric oxide (NO).

Methods

Cell Culture

The human breast adenocarcinoma cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Manassas, VI, USA) and cultured in Dulbecco's modified Eagle's medium and F12 medium (DMEM:F12; 1:1; Invitrogen Inc., Carlsbad, CA, USA) with 2 mM L-glutamine, 80 μg/ml gentamycin, supplemented with 10% heat-inactivated fetal calf serum (FCS; PA/Bioser, Bioser S.A., Barcelona, Spain) at 37°C in a humidified 5% CO_2 air. MCF-10A cells were also purchased by ATCC and are not a tumorigenic cell line derived from a human female mammary reduction. Cells were grown on tissue culture plastic dishes, in DMEM:F12 supplemented with 10% FCS, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), and epidermal growth factor (20 ng/ml). Both cell lines were detached using the following buffer: 0.25% trypsin and 0.02% EDTA in Ca^{2+} and Mg^{2+}-free phosphate buffer saline (PBS) of confluent monolayers. The medium was replaced three times a week. Cell viability was assayed by Trypan blue exclusion test, and the absence of mycoplasma was confirmed by Hoechst staining [15].

IgG Purification

Cancer patients with breast adenocarcinoma at stage I were selected in the Mastology Department of the Angel H. Roffo Oncology Institute, University of Buenos Aires, and were classified according to TNM criteria as T1N0Mx (tumor size <2 cm, without axillary node metastases). All of them were free of treatment, and blood samples (10–20 ml/donor) were obtained at routine procedures before surgery. Subjects free of illness (normal) or with breast benign fibroadenoma (BFA) were used as controls. Informed consent and acceptance was obtained from each patient. After centrifugation, sera were heat-inactivated at 56°C for 30 min. IgG purification was performed by affinity chromatography in protein G-agarose (Invitrogen). Samples (pH 6–7.5) were loaded onto the column after equilibrating it with 10 volumes of binding buffer, 0.01 sodium phosphate pH 7.0, 0.15 M NaCl at high salt concentration. Elution was performed with 0.1 M glycine hydrochloride (pH 2.6) and adjusted immediately to pH 7.0. Concentration of protein in the collected fractions was obtained by measuring the absorbance at 280 nm, and fractions that corresponded to the peak of maximal absorbance were stored at −20°C. IgG numbers indicate samples obtained from different patients with breast tumors in T1N0Mx.

Cell Proliferation Assay

Growth studies in vitro were conducted using the soluble tetrazolium salt MTS colorimetric assay (Cell Titer 96TM AQueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI, USA). MTS couples fenazine methylsulfate (PMS) and is reduced to formazan. MCF-7 cells were seeded in 96-well plates at a density of 10^4 cells per

well in DMEM:F-12 medium supplemented with 5% FCS and were left to adhere overnight. Subconfluent conditions (about 20–30%) were chosen to allow detection of maximal growth. Then, cells were deprived of FCS to induce quiescence (24 h previous to the proliferation assay) and were treated with increasing concentrations of CARB or with the maximal effective concentration (10^{-9} M) of the agonist, in triplicate during 1 h in the absence or presence of different muscarinic antagonists (10^{-6} M): atropine, 4-diphenylacetoxi-N-methylpiperidine (4-DAMP), tropicamide or different enzymatic inhibitors: 10^{-6} M 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC),10^{-4} M N^Gmonomethyl-L-arginine (L-NMMA), 10^{-3} M aminoguanidine, that were added 30 min previous to CARB. IgG from normal donors, BFA patients, or T1N0Mx cancer patients were assayed by triplicate at different concentrations for 1 h in the absence or presence of antagonists or enzymatic inhibitors mentioned above. After treatment, medium was replaced by fresh medium free of FCS and cultured during 48 h. Supernatants were discarded, and viable cells were detected by adding 20 μl MTS:PMS (20:1) to each well, and after 2 h of incubation at 37°C, the production of formazan was evaluated by measuring the absorbance at 490 nm with an ELISA reader (Bio-Rad Laboratories, Inc. Oakland, CA, USA). Mean absorbance value of triplicate samples ± SEM were calculated, and results were expressed as percent of stimulation in relation to control (cells without treatment).

Detection of mAChR Subtypes by Western Blot

MCF-7 or MCF-10A cells (2×10^7) were washed twice with PBS and lysed in 1 ml of modified RIPA buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 5 mM NaF, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM PMSF, 1% Triton X-100, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After 1 h in ice bath, lysates were centrifuged at 10,000 rpm for 20 min at 4°C [13]. Resulting supernatants were stored at −80°C, and protein concentration was determined by the method of Bradford [16].

MCF-7 or MCF-10A cell lysates (30 μg protein/lane) were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) minigels electrophoresis. Then, proteins were transferred to nitrocellulose membranes (Bio-Rad) and washed with distilled water. The nitrocellulose strips were blocked in 20 mM Tris–HCl buffer, 500 mM NaCl, and 0.05% Tween 20 (TBST) with 5% skimmed milk for 1 h at 20°C to 25°C and subsequently incubated overnight with goat anti-M_1, anti-M_2, and anti-M_3 polyclonal or with rabbit anti-M_4 and anti-M_5 Abs (all at 1:100; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) in TBST. After several rinses with TBST, strips were incubated with the second antibody (rabbit or goat anti-mouse IgG conjugated with alkaline phosphatase 1:4,000; Sigma Chemical Co. St. Louis, MO, USA) in TBST at 37°C during 1 h. Bands were visualized with a mixture of nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt [13].

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Quantification of the bands was performed by densitometric analysis using Image J program (NIH) and was expressed in optical density units relative to GAPDH.

NO Production

NO production by NOS was tested by measuring nitrite (NO_2^-) accumulation in culture supernatants. MCF-7 cells (10^4/well) were seeded in triplicate in 96-well plates with 100 μl of DMEM:F-12 (Invitrogen) plus 10% FCS. Then, fresh medium without FCS was added, and cells were treated with CARB (10^{-9} M) or IgG (2 μg/ml) in triplicate, during 1 h in the absence or presence of muscarinic antagonists (10^{-6} M): atropine, 4-DAMP, and tropicamide or enzymatic inhibitors, 10^{-6} M NCDC, 10^{-4} M L-NMMA, 10^{-3} aminoguanidine, which were added 30 min previous to CARB. After treatment, medium was replaced by fresh medium free of FCS. NO_2^- production was evaluated after 24 h in culture supernatants by Griess reagent [1% sulphanylamine in 30% acetic acid with 0.1% N-(1 naphtyl) ethylenediamine in 60% acetic acid]. Absorbance was measured at 540 nm with an ELISA Reader (Bio-Rad). NO_2^- concentration was calculated with respect to a standard curve of $NaNO_2$ diluted in culture medium and was expressed in micromolar concentration (μM) [17].

Detection of NOS Isoforms by Western Blot

MCF-7 cells (2×10^7) were washed twice with PBS and lysed in 1 ml of modified RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4; 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 5 μg/ml aprotinin and leupeptin). After 1 h in ice bath, lysates were centrifuged at 10,000 rpm for 20 min at 4°C [14]. Resulting supernatants were stored at −80°C, and protein concentration was determined by the method of Bradford [16].

MCF-7 cell lysates (30 μg protein/lane) were prepared as it was previously described and were subjected to 7.5% SDS-PAGE electrophoresis. Then proteins were transferred to nitrocellulose membranes (Bio-Rad) and washed with distilled water. The nitrocellulose strips were blocked in TBST with 5% skimmed milk for 1 h at 20°C to 25°C and subsequently incubated overnight with rabbit anti-NOS1 or anti-NOS3 or goat anti-NOS2 polyclonal Abs (all at 1:100; Santa Cruz Biotechnology). After several rinses with TBST, strips were incubated with the second antibody (rabbit or

goat anti-mouse IgG conjugated with alkaline phosphatase all at 1:4,000; Sigma Chemical Co) in TBST at 37°C during 1 h. Bands were visualized by ECL [18].

Drugs

All drugs were purchased from Sigma Chemical Co (St. Louis, MI, USA) unless otherwise stated. Solutions were prepared fresh daily.

Statistics

Results are given as means ± SEM of at least three independent experiments. The statistical significance of differences between groups was analyzed by analysis of variance using GraphPad Prism 4; $p<0.05$ was considered to be statistically significant.

Results

We had previously reported that human tumor breast samples, surgically obtained from mammary adenocarcinomas, overexpress M_3 and M_2 receptors, while mAChR are absent in normal breast human tissue (obtained from mammary reduction procedures). As it is shown in Fig. 1a, MCF-7 cells, derived from a human breast adenocarcinoma, express M_3 and M_4 receptor subtypes, while MCF-10A cells, a normal human breast cell line, do not express any of the mAChR subtypes tested (Fig. 1b). Then, we assayed the function of these receptors by stimulating them with increasing concentrations of the muscarinic agonist CARB. It can be seen in Fig. 1c that CARB exerts a concentration-response effect on tumor cells proliferation, being 10^{-9} M the maximal effective concentration of the agonist that increased MCF-7 cells growth by 48±5%. This effect was reverted by preincubating cells with the nonselective muscarinic antagonist atropine (10^{-6} M) or by the M_3 selective antagonist, 4-DAMP (10^{-6} M), while 10^{-6} M tropicamide, an M_4 selective antagonist, failed to reduce CARB action. Table I shows that muscarinic antagonists lack of effect on MCF-7 cells proliferation because they did not modify the absorbances in comparison with control (untreated cells) measured at 490 nm in MTS assays.

We also demonstrated that because of the absence of mAChR, MCF-10A cells did not respond to CARB at any concentration tested (Fig. 1d).

Taking into account that we had described the existence of an IgG fraction that recognized and activated mAChR in murine mammary adenocarcinoma bearers, we tested the presence of a similar fraction in three human female breast cancer patients. In similar experiments to those performed with the pharmacological agonist, we assayed the action of different concentrations of IgG (0.2–200 μg/ml) purified from patients in stage I. Figure 2a shows the stimulatory effect exerted by T1N0Mx-IgG on MCF-7 cells proliferation, ranging from 17±2% to 58±7%. This effect was significantly reduced by preincubating cells with 10^{-6} M atropine.

We also tested the action of IgG purified from BFA patients ($n=3$), and we observed that it exerted a slight stimulation on MCF-7 cells proliferation effect that was also reduced by atropine (Fig. 2b). Considering the sensitivity and the reproducibility of the method used to measure cell proliferation, we arbitrary determined that the action triggered by IgG was significant if it was higher than 20% with respect to control (untreated cells). IgG obtained from patients with BFA did not modify tumor cells proliferation in a significant manner. Thus, we observed that IgG from normal donors ($n=3$) was able to stimulate proliferation over the cut-off value from 2 μg/ml. This effect was prevented by atropine up to 20 μg/ml (Fig. 2c). IgG from cancer patients, from BFA patients, or from normal donors did not modify the proliferation of MCF-10A cells either in the absence or in the presence of atropine (data not shown).

In order to investigate which mAChR subtype is involved in the stimulatory action of T1N0Mx-IgG on MCF-7 cells proliferation, we performed experiments in the presence of selective muscarinic antagonists. As it is shown in Fig. 3a, the action of the maximal effective concentration of CARB (10^{-9} M) was reduced by (10^{-6} M) atropine and 4-DAMP but not by tropicamide (10^{-6} M).

When we tested the action of IgG purified from five patients with breast cancer in T1N0Mx (at the maximal effective concentration 2 μg/ml) on MCF-7 cells proliferation, we observed that all of them significantly stimulated tumor cells growth. Although 10^{-6} M 4-DAMP added before T1N0Mx-IgG reduced the effect in four of five studied patients, we observed that tropicamide at the same concentration also diminished IgG stimulatory action in four of five patients indicating that M_4 receptors could be also involved in the action of cancer patients IgG (Fig. 3b). The effect of IgG from normal donors at 2 μg/ml also rose over the cut-off value and was reduced by 4-DAMP but not by tropicamide (Fig. 3c).

Stimulation of the M_3 receptor subtype is usually linked via Gq/11 protein to PLC-NOS pathway activation. We observed that the effect of CARB (10^{-9} M) on MCF-7 cells proliferation (48±5%) was significantly reduced by preincubating cells with 10^{-6} M NCDC or by 10^{-4} M L-NMMA, pointing to the participation of PLC and NOS-derived NO in this effect (data not shown).

We also demonstrated that the addition of increasing concentrations of CARB stimulated NO formation, being

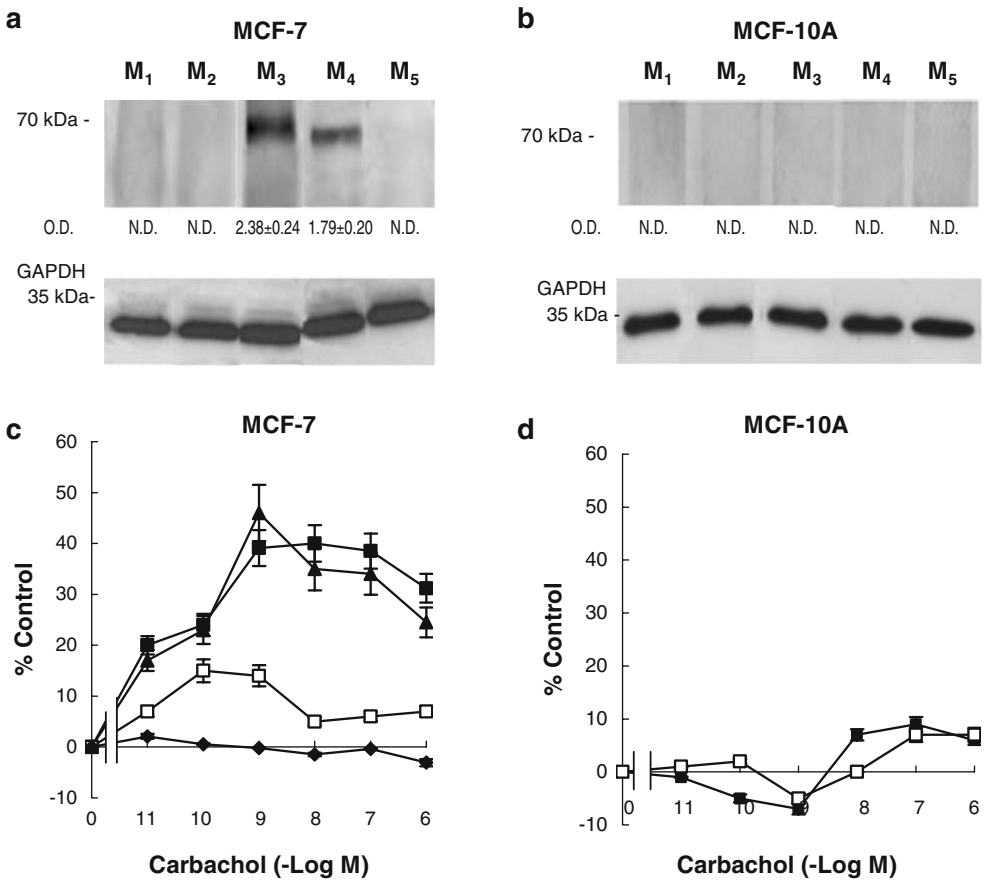

Fig. 1 Muscarinic acetylcholine receptors (mAChR) expression and function. Western blot experiments to detect mAChR subtypes were performed in lysates from (**a**) MCF-7 cells and (**b**) MCF-10A cells. Molecular weights of proteins are indicated on the *left*. Optical density of the bands was calculated by densitometric analysis, and values were relativized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was used as loading control. *ND* not detectable. One representative experiment of three is shown. Values are mean ± SEM of three experiments. **c** Concentration-response curves of carbachol in the absence (*closed squares*) or in the presence of 10^{-6} M atropine (*open squares*), 4-DAMP (*closed diamonds*), or tropicamide (*closed triangles*) on MCF-7 cells proliferation. **d** Concentration-response curves of carbachol in the absence (*closed squares*) or in the presence of atropine (*open squares*) on MCF-10A cells proliferation. Values are mean ± SEM of six experiments performed in triplicate

10^{-9} M CARB the maximal effective concentration for this effect (Basal, 1.98±0.20 μM; CARB, 6.80±0.71 μM; $p<$ 0.01; Fig. 4c). By Western blot experiments, we demonstrated the expression of calcium-dependent isoforms, NOS1 and NOS3, either in MCF-7 tumor cells or in MCF-10A normal cells (Fig. 4a, b). The participation of mAChR in NO production by tumor cells was evidenced by the addition of 10^{-6} M atropine that significantly reduced the action of CARB (Fig. 4c).

In addition, 10^{-6} M 4-DAMP also reduced CARB-induced NO synthesis, while tropicamide (10^{-6} M) failed to modify CARB action on NO liberation (Fig. 4c). Although MCF-10A cells produce higher amounts of NO, they were not able to respond to CARB at any concentration tested (Fig. 4d). In addition, we observed that in tumor cells, the action of the maximal effective concentration of CARB on NO synthesis was reverted by the addition of 10^{-6} M NCDC (3.90±0.30 μM) or 10^{-4} M L-NMMA (3.50± 0.29 μM) previous to CARB. Aminoguanidine (10^{-3} M) did not modify CARB action on NO synthesis (Fig. 5a). Our experiments indicate that CARB-derived NO synthesis was due to M_3 receptors activation, since 4-DAMP

Table I Effect of Muscarinic Antagonists and Enzymatic Inhibitors on MCF-7 Cells Proliferation

	Control	AT	4-DAMP	TROP
Absorbance	0.594±0.015	0.508±0.062	0.519±0.045	0.579±0.037

The addition of 10^{-6} M: atropine (AT), 4-diphenylacetoxi-N-methyl-piperidine (4-DAMP), and tropicamide (TROP) did not modify tumor cell proliferation in comparison with control (cells without treatment). Absorbances for MTS reaction were measured at 490 nm and are mean ± SEM of $n=5$ experiments performed in triplicate

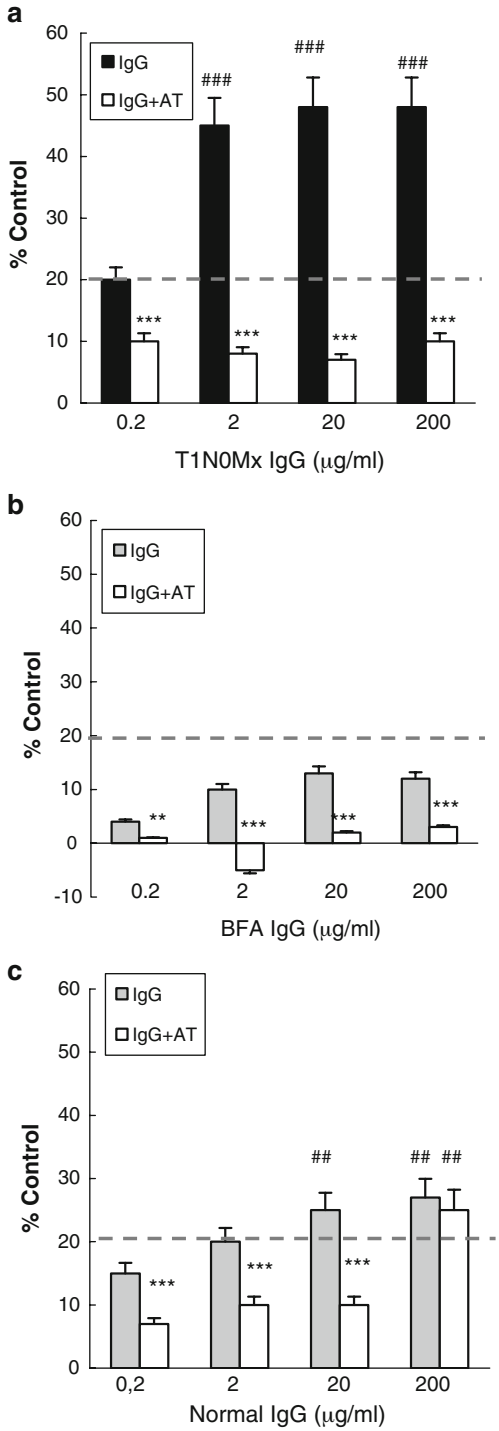

Fig. 2 Effect of IgG from breast cancer patients on MCF-7 cells proliferation. Concentration-response curves of (**a**) T1N0Mx IgG (*black bars*), (**b**) benign fibroadenoma (BFA) IgG (*gray bars*), and (**c**) normal IgG (*gray bars*) in the absence or presence of atropine (AT; 10^{-6} M). Values are mean ± SEM of three experiments performed in triplicate. ###p<0.001, ##p<0.01 vs. cut-off value. ***p<0.001, **p <0.01 vs. without AT

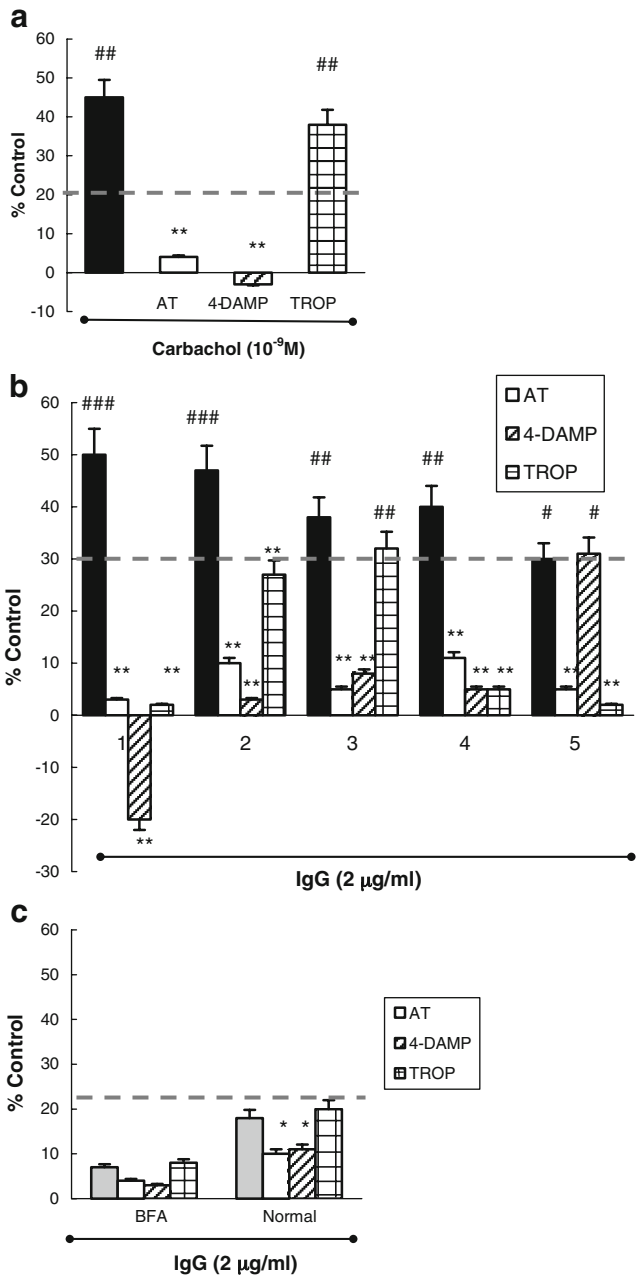

Fig. 3 Action of the maximal effective concentration of carbachol or T1N0Mx IgG on MCF-7 cells proliferation. MCF-7 cells were treated with (**a**) carbachol (10^{-9} M), (**b**) T1N0Mx-IgG (2 μg/ml), (**c**) normal or benign fibroadenoma (BFA) IgG (2 μg/ml) in the absence or presence of 10^{-6} M: atropine (AT; not selective mAChR antagonist), 4-DAMP (M_3 receptor selective antagonist), or tropicamide (TROP; M_4 selective antagonist). Values are mean ± SEM of three experiments performed in triplicate. ###p<0.001, ##p<0.01 vs. cut-off value. **p<0.001, *p< 0.01 vs. carbachol or T1N0Mx-IgG

significantly reduced the agonist action (4-DAMP+ CARB:2.00±0.23 μM). Tropicamide was not effective to reduce CARB-stimulated NO synthesis by tumor cells (tropicamide+CARB:7.00±0.65 μM; Fig. 5a). Muscarinic

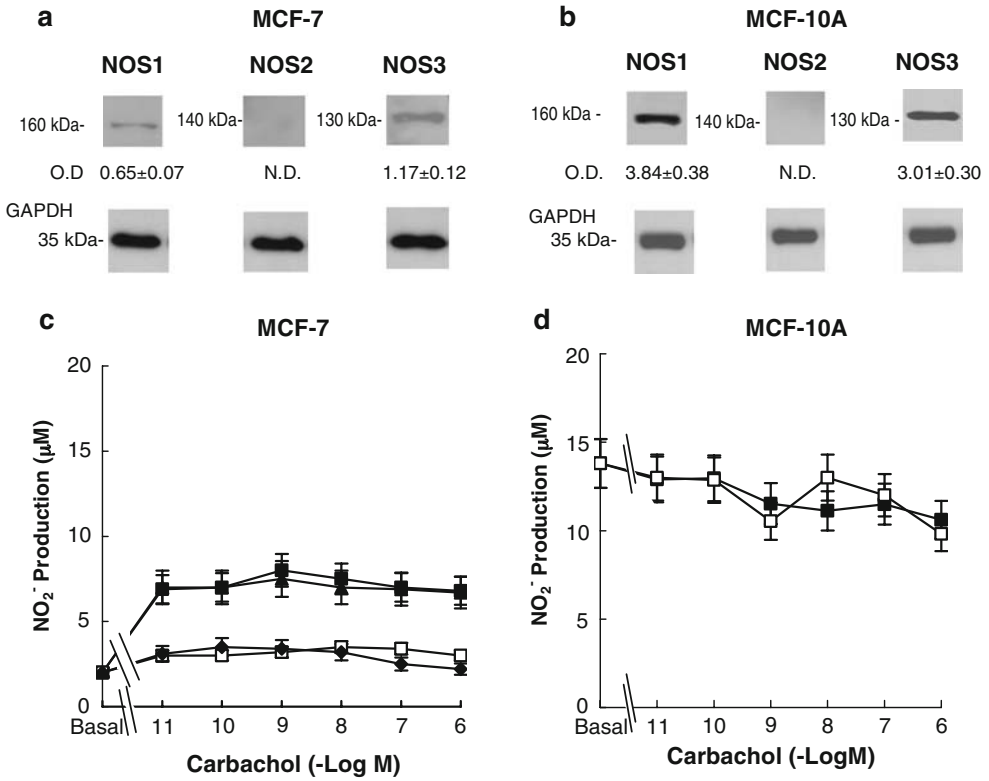

Fig. 4 Nitric oxide synthase (NOS) expression and function. Western blot experiments to detect NOS isoforms were performed in lysates from (**a**) MCF-7 cells and (**b**) MCF-10A cells. Molecular weights of proteins are indicated on the *left*. Optical density (OD) of the bands was calculated by densitometric analysis, and values were relativized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was used as loading control. *ND* not detectable. One representative experiment of three is shown. Values are mean ± SEM of three experiments. **c** Concentration-response curves of carbachol in the abscense (*closed squares*) or in the presence of 10^{-6} M: atropine (*open squares*), 4-DAMP (*closed diamonds*), or tropicamide (*closed triangles*) on nitrite (NO_2^-) production in micromolar (μM) concentration by MCF-7 cells. **d** Concentration-response curves of carbachol in the absence (*closed squares*) or in the presence of 10^{-6} M atropine (*open squares*) on NO_2^- production by MCF-10A cells. Values are mean ± SEM of three experiments performed in triplicate

antagonists or enzymatic inhibitors did not modify NO production in MCF-7 cells, when they were tested alone (Table II).

We also tested the action of IgG from T1N0Mx patients on NO synthesis by tumor cells, and we evidenced that all of them were able to stimulate NO production at 2 μg/ml in a more potent manner than the agonist. This action was prevented in all cases by atropine, revealing muscarinic participation in this effect (Fig. 5b). Moreover, preincubation of tumor cells with 10^{-6} M 4-DAMP reduced IgG derived-NO liberation induced by the action of four of five T1N0Mx IgG. Tropicamide at the same concentration also diminished IgG stimulatory action induced by four of five IgG from cancer patients, indicating that both M_3 and M_4 receptors are also involved in T1N0Mx-IgG action on NOS activity (Fig. 5b). Although BFA or normal IgG could increased basal NO liberation in MCF-7 cells, these effects were not due to mAChR stimulation because they were not modify by atropine, 4-DAMP, or tropicamide.

Discussion

Acetylcholine is the main neurotransmitter in the central and parasympathetic nervous system. In the last years, several evidences indicated that acetylcholine, its receptors (nicotinic and muscarinic), and the enzymes that synthesize and degrade it are present in cells without nervous origin conforming the non-neuronal cholinergic system (nNCS). It has been documented the role of nNCS in the regulation of important cell functions as mitosis, cell morphology, locomotion, and immune response that are key steps during tumor progression [19].

We had previously reported the expression of high concentrations of mAChR in three cell lines: LM2, LM3, and LMM3, derived from different murine mammary adenocarcinomas and the absence of these receptors in the normal cell line NMuMG. The activation of mAChR in these murine mammary tumor cells also promoted cells proliferation [13]. Several evidences demonstrated over-expression of mAChR in different type of tumor tissues

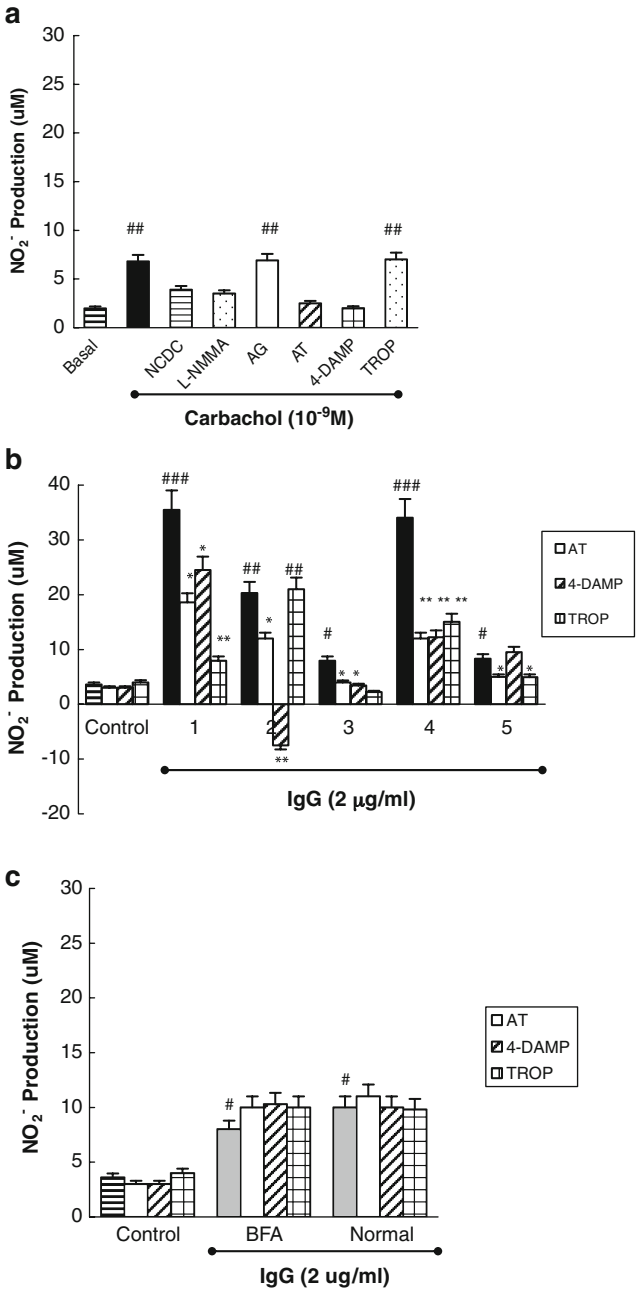

Fig. 5 Action of the maximal effective concentration of carbachol or T1N0Mx IgG on nitric oxide synthase activity measured as nitrite (NO_2^-) production expressed in micromolar (μM) by MCF-7 cells treated with (**a**) carbachol (10^{-9} M) in the absence or presence of 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC; 10^{-6} M), N^Gmonomethyl-L-arginine (L-NMMA; 10^{-4} M), aminoguanidine (AG; 10^{-3} M), 10^{-6} M: atropine (AT), 4-DAMP, or tropicamide (TROP) on NO_2^- production; with (**b**) T1N0Mx-IgG (2 μg/ml); or with (**c**) normal or benign fibroadenoma (BFA) IgG (2 μg/ml) in the absence or presence of 10^{-6} M: atropine (AT), 4-DAMP, or tropicamide (TROP). Values are mean ± SEM of three experiments performed in triplicate. ###$p<0.001$, ##$p<0.01$, #$p<0.05$ vs. basal (untreated cells). **$p<0.001$, *$p<0.01$ vs. T1N0Mx-IgG

derived from brain, colon, prostate, lung, skin, and uterus, and we also described the presence of M_3 and M_2 receptor subtypes in human breast tumor tissue and the absence of them in normal breast tissue [20, 21].

The presence of auto-Abs that recognizes and activates mAChR was described in different human pathologies as Chagas disease, Sjögrem syndrome, and AIDS patients [22–24]. There is an important parallel between cancer and the systemic autoimmune diseases, both are characterized by a diverse collection of auto-Abs directed against different antigens. Most of the antigens identified in human tumors are self-proteins, without mutations but inappropriately expressed or overexpressed. As a consequence, instead of acting as tumor-rejecting antigens, auto-Abs induce tolerance or autoimmune responses to normal tissue [25]. Although we observed that IgG from breast cancer patients exerts a stimulatory concentration-dependent effect on MCF-7 cells proliferation via mAChR activation mimicking CARB action (Fig. 2a), we observed differences between the magnitude of CARB action and T1N0Mx-IgG action on MCF-7 cells proliferation. The fact that T1N0Mx-IgG exerts a more potent action than CARB could be due to different mechanisms of action that include different coupling to effectors that triggers the signal transduction pathways and/or the dimerization or oligomerization of receptors with stabilization and permanent activation promoting tumor cells proliferation. Schulze W. et al. [26] have reviewed the binding of the auto-Abs to epitopes of β1 or M_2 receptors that belong to the GPCR family in various cardiovascular diseases. In spite this, interaction is not completely understood; it has been shown that agonistic stimulation by auto-Abs shifts the receptor to a dimeric state, stabilizes this conformation, and activates the signal cascade. They assumed that the agonistic auto-Abs trigger an active dimeric conformation in GPCR. In contrast to the physiologically regulated signal cascade, the auto-Abs binding leads to a lack of receptor downregulation and a permanent overstimulation of the GPCR. It has also been documented that GPCR might form homo- or heterodimers. Issafras et al. [27] have described that CCR5 receptors exist as constitutive oligomers and, as it was demonstrated in the case of the $GABA_B$ receptor, heterodimerization and homodimerization might play a role in endoplasmic reticulum export quality control. They also show that Abs against CCR5, that also belongs to GPCR family, cause the clustering of preexisting oligomers. Regarding proliferation and NO synthesis in our model, it could be possible that T1N0Mx-IgG exerts the clustering of M_3 and M_4 receptors, triggering and magnifying the signaling pathways of both receptors, and because of this, responses that are quantitative higher than those produced by the agonist could be antagonized by tropicamide or 4-DAMP.

Table II Effect of Muscarinic Antagonists and Enzymatic Inhibitors on Nitric Oxide Production by MCF-7 Cells

	Control	NCDC	L-NMMA	AG	AT	4-DAMP	TROP
Absorbance (10^{-2})	7.65±1.01	7.48±2.10	8.07±0.71	7.90±2.8	8.00±2.20	7.65±1.60	7.80±1.00

The addition of 10^{-6} M 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC), 10^{-4} M N^G monomethyl-L-arginine (L-NMMA), 10^{-3} M aminoguanidine (AG) or 10^{-6} M: atropine (AT), 4-diphenylacetoxi-N-methylpiperidine (4-DAMP), tropicamide (TROP) did not modify the production of nitric oxide measured as nitrite in comparison with control (cells without treatment). Absorbances for Griess reaction were measured at 540 nm, and values are mean ± SEM of $n=5$ experiments performed in triplicate

The role of mAChR subtypes expression and function in cancer led at the beginning to contradictory evidences. While, published results demonstrated that the activation of M_3 receptors inhibited proliferation of small cells lung carcinoma, a great deal of evidences suggested that acetycholine secreted from these cells promotes lung carcinoma progression, acting as an autocrine growth factor [28, 29]. Our results are in accordance with the latter, because CARB added at low concentrations (10^{-9} M) is able to stimulate MCF-7 cells proliferation by activating mAChR, particularly, the M_3 subtype (Fig. 3a). This receptor subtype is linked to tumor cells proliferation either in animal models or in humans. Raufman et al. [30] have reported that colon epithelial cells and most colon cancers overexpress M_3 receptors. In human colon cancer cells, post-M_3 signaling stimulates tumor cells proliferation, and M_3-deficient mice attenuated epithelial cell proliferation and tumor number and size. We demonstrated that major expression of M_3 receptor subtype is involved in murine mammary malignant LMM3 tumor growth, because CARB-stimulated proliferation is totally reverted by pre-incubating cells with the M_3 selective antagonist, pf-HHSiD [31]. This function is generally linked to calcium influx into the cells triggered by M_3 receptor activation via PLC; we confirmed this effect blunting CARB action on proliferation with NCDC, a PLC inhibitor. Preliminary experiments have shown us that CARB is able to trigger inositol phosphate-1 accumulation via PLC activation in MCF-7 cells, an effect that was reverted by 4-DAMP (data not shown).

It must be considered that breast tumors exhibit a modulation in mAChR subtypes expression during progression by modifying the ratio M_3/M_4 that could be determining the concentration, affinity, and specificity of anti-mAChR Abs in each cancer patient. We also observed that the action of T1N0Mx-IgG was generally reverted with tropicamide (Fig. 3b). M_4 receptor activation couples Gi protein to adenylyl cyclase and inhibits cAMP formation. O'Shaughnessy et al. [32] demonstrated that the addition of AMPc to T lymphocytes cultures inhibits mitosis. In tumor cells, a cross-talk between cAMP/protein kinase A and Ras pathways has been described, and authors showed that the activation of cAMP/protein kinase A metabolic pathway inhibited human transformed tyrodes cells and promoted apoptosis [33]. Then, it could be possible that the inhibition of M_4 receptor signaling pathway facilitates proliferative actions exerted by M_3 receptor activation. Although the functional specificity of mAChR is frequently summarized as the M_1, M_3, and M_5 receptors mediating activation of PLC via Gq protein, while not inhibiting adenylyl cyclase, and the M_2 and M_4 receptors mediating inhibition of adenylyl cyclase via Gi protein, without stimulating PLC, this specificity is not absolute because M_2 and M_4 receptors can weakly couple to PLC when expressed at high levels in certain cell types [34].

We also observed that normal IgG exerted muscarinic proliferative action on MCF-7 cells, though the effect was lower than that produced by T1N0Mx-IgG (Fig. 2c). Abs that bind to a variety of exogenous antigens, as well as self-antigens (i.e., nucleic acids, phospholipids, and cellular components), account for a significant proportion of immunoglobulin in healthy individuals. These Abs that react with self-molecules occur in healthy individuals and are referred to as natural Abs or auto-Abs, because they arise independently of known and/or deliberate immunization. Because of their broad reactivity for a wide variety of microbial components, natural Abs have a major role in the primary line of defense against infections. Since they also recognize a variety of self-antigens, they have a role in the development of the B-cell repertoire and the homeostasis of the immune system [35]. We cannot discard the presence of natural Abs against mAChR in healthy donors.

NO is a pleiotropic diatomic radical that plays a variety of regulatory functions as a neurotransmitter, a vasoactive mediator, and regulator of numerous physiological functions. In addition, NO is considered an important mediator in carcinogenesis. This molecule is produced by the three isoforms of NOS. Despite these beneficial effects, some reports suggest that NO exerts antitumor properties, while others implicate NO in tumor promotion, depending on its local concentration. NO liberated by calcium-dependent isoforms, NOS1 and NOS3, promotes tumorigenesis, while NOS2-derived NO generally induces antitumor actions, cell cycle arrest, and apoptosis [36]. Both MCF-7 and MCF-10A cells express NOS1 and NOS3 isoenzymes that produce NO at concentrations that should be favoring cell proliferation (Fig. 4a, b), but tumor cells differ from normal

cells in the expression of mAChR that could be a switch-on mechanism to activate NO synthesis. It is well known that M_3 receptor activation leads to calcium mobilization via PLC and as a consequence to the activation of different enzymes as NOS [7]. The maximal effective concentration of CARB that stimulates NO synthesis by NOS is coincident with that one that stimulates tumor cells proliferation (Fig. 4c). The participation of PLC-NOS pathway in CARB-induced NO production was demonstrated because the PLC inhibitor NCDC and L-NMMA, a NOS nonselective inhibitor, reverted the agonist action (Fig. 5a). We also confirmed that NO was produced by the calcium-dependent isoenzymes NOS1 and NOS3, because aminoguanidine, an NOS2 selective inhibitor, was unable to reduced CARB-induced NO synthesis (Fig. 5a). T1N0Mx-IgG also stimulated NOS activity in MCF-7 cells, producing higher amounts of NO than CARB (Fig. 5b). Other authors have reported the presence of circulating Abs from Sjögren syndrome that interact with mAChR expressed in rat cerebral frontal cortex and activate them. IgG from patients with this syndrome displayed an agonistic-like activity associated to specific M_1 and M_3 mAChR activation, increasing NOS1 and NOS3 isoforms activity expressed in cerebral frontal cortex [37]. Our results indicate that T1N0Mx-IgG triggered NO liberation via M_3 receptor activation, but the effect of IgG from four of five selected patients on NO synthesis was also prevented by M_4 receptor antagonism (Fig. 5b). It has been shown that the activation of both M_2 and M_4 receptors in Chinese hamster ovary cells that co-express the individual receptor subtypes and eNOS in a stable fashion resulted in marked activation of eNOS in a time- and concentration-dependent manner [38]. This could be revealing that M_4 receptor in MCF-7 cells is linked to NOS signaling pathway. In conclusion, IgG from breast cancer patients in T1N0Mx activates tumor cells proliferation via mAChR activation and NO production, effect that could be promoting early tumor growth in these patients. Detection of these auto-Abs could be a useful tool of prognosis in this illness.

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