Research Journal of Biological Sciences 3 (2): 246-250, 2008
ISSN: 1815-8846
© Medwell Journals, 2008

Atrial Natriuretic Factor Downregulation During Breast Cancer Progression

Biagio Valentino, Giampiero La Rocca, Rita Anzalone, Simona Corrao, Francesca Rappa, Diego Lipari, Alessandro Valentino, Giovanni Peri, Francesco Cappello and Elvira Vittoria Farina-Lipari
Department of Experimental Medicine, Section of Human Anatomy, University of Palermo, Italy

Abstract: We researched in a series of human breast cancers of different stages the presence and the expression of Atrial Natriuretic Factor (ANF) and Oxytocin (OX), by immunohistochemistry and RT-PCR. We found a reduction of expression of ANF during cancer progression. By contrast, OX resulted lightly expressed by immunohistochemistry and negative at RT-PCR. We hypothesize that ANF could be considered a novel marker to predict breast cancer progression; by contrast, OX expression should be better investigated, to clarify also the putative role of oxytocinases in human breast carcinogenesis.

Key words: Atrial natriuretic factor, oxytocin, breast cancer, carcinogenesis

INTRODUCTION

The breast gland is composed of between 15 and 25 lobes each empting into a separate major duct terminating in the nipple. Each lobe is surrounded of connective tissue and is divided into many lobules. The lobules are subdivided into 10-100 alveoli which are enveloped by a true basal lamina (Haagensen, 1986). Under hormonal influence, especially sex steroids, the alveolar epithelium specializes into A, B and myoepithelial cells. B cells are thought to be precursors of columnar, basophilic luminal A cells. Myoepithelial cells, located in close contact with plasma membrane of alveolar epithelium, are 10-20 times more sensitive to Oxytocin (OX) than myometrial cells (Vorherr, 1974).

An epithelial-stromal junction surrounds the epithelium, delimiting it from a fibroblast-rich connective tissue (McCarty *et al*., 1983). Histologic features vary with development and menstrual cycling, as well as menopause. Indeed, the declining levels of sexual hormones are associated with microenviromental modification in mammary cell trophism that is proved by breast involution (Vorher, 1980).

Breast Cancer (BC) is one of the most commonly diagnosed carcinomas in the World and one of the leading causes of cancer-related death in women. BC are divided into in situ and invasive carcinomas. Both are classified as ductal and lobular on the basis of their histologic features (Wellings, 1980). Because of the growth of BC is often regulated by the female sex steroids, the immunohistochemical determination of their cellular concentration is used to predict which patients may benefit from antihormonal therapy (Elledge and Fuqua, 2000). Nevertheless, the roles of other paracrine factors that may directly or indirectly modulate signal transduction pathways during breast carcinogenesis have not been widely studied to date (Dickon and Russo, 2000).

OX is a cyclic nonapeptide synthesized as a larger precursor molecule that is rapidly converted by proteolysis to the active hormone (Zingg *et al*., 1998). Well demonstrated sites of OX synthesis include hypothalamic supraoptic nucleus, ovarian luteal cells, endometrium and placenta (McCracken *et al*., 1999; Egarter and Husslein, 1992). OX plays many important physiological roles in uterus during pregnancy and breast during lactation (O'Brien, 1995). Moreover, we found OX presence in human prostate gland, simultaneously to Atrial Natriuretic Factor (ANF), and postulated that OX and ANF may have paracrine effects in the homeostasis of this gland (Farina-Laipari *et al*., 2003). Indeed, ANF has endocrine/paracrine functions in many districts, acting on renal excretion of sodium and water and relaxation of vascular smooth muscle cells (Currie *et al*., 1983). Moreover, ANF inhibits of aldosterone synthesis from adrenal cortical cells (Atarashi *et al*., 1984), vasopressin release from posterior pituitary gland (Samson, 1985) and renin production from renal cortical cells (Burnett *et al*., 1984), interestingly, ANF has also paracrine/autocrine functions, i.e., during spermatogenesis in rats (Pandey, 1991).

We have already demonstrated the presence of ANF in the excretory ducts of the rabbit parotid gland (Valentino *et al*., 1999), postulating the possible paracrine role of this peptide in cell homeostasis mechanisms, as the regulation of the salivary composition. Moreover, a number of papers have already postulated the functional

Corresponding Author: Dott. Francesco Cappello, Via alla Falconara 120, 90136, Palermo, Italy

interaction between OX and ANF in several organs, as heart (Faveretto *et al*., 1997), kidney (Soares *et al*., 1999), myometrium (Carrajal *et al*., 2001) and prostate (Farina-Laipari *et al*., 2003). In addition, we recently showed the presence of both ANF and OX in non-lactating breast gland of rabbit (Valentino *et al*., 2005), hypothesizing also that these hormones may be involved in homeostasis of breast parenchyma.

In the present study, we researched the presence and the expression of OX and ANF by immunohistochemical and biomolecular techniques in a series of BCs of different stages of progression, to investigate the involvement of these hormones during mammalian carcinogenesis.

MATERIALS AND METHODS

Specimen collection: We collected from the Department of General Surgery of the "Policlinico P. Giaccone" of the University of Palermo 24 specimens of ductal carcinoma of different stages. In particular, 6 specimens were of in situ ductal Carcinoma (CIS), 13 specimens of N0 ductal carcinoma (N0) and 11 specimens of N1 ductal carcinoma (N1). Moreover, we collected 10 biopsies of Non-tumoral Breast tissue (NB). Tissues were in part formalin-fixed and paraffin-embedded for immunohistochemical analyses; moreover, a small specimen of each NB, N0 and N1 was frozen for western blotting and RT-PCR studies, as described below.

Immunohistochemistry: Immunostaining by streptavidin-biotin complex method (LSAB2 kit peroxidase, DAKO Corporation, Carpinteria CA, Cat. No K0677) was performed, using monoclonal antibodies against ANF at the dilution of 1:500 (Cymbus Biotechnology LTD, Cat. No. CBL66) and OX at the dilution of 1:1000 (Chemicon, Cat. No. AB911) and isotype-matched control on 5-micra formalin-fixed paraffin-embedded sections. After incubation for 10 min with protein block serum-free (DAKO Corporation, Carpinteria CA, Cat. No X0909), the primary antibody was added to the sections. DAB was used as develop chromogen (DAKO Corporation, Carpinteria CA, Cat. No K3467). Results were semiquantitated by 2 independent observers (FR and FC), according to the cell percentage presenting immunopositivity on 10 HPF. The mean of the values were considered as data. We analysed the significance of the data using the Student "t" test ($p<0.05$). A one-way Analysis of Variance (ANOVA) was used to determine the correlation between either ANF and OX expression and tumour stage.

Total RNA extraction: Total RNA extraction was accomplished using the QuickPrep Total RNA Extraction Kit (Amersham Biosciences, Milan) following the manufacturer's instructions. RNA yield was evaluated spectrophotometrically (A260/A280) and RNA aliquots were stored at -80°C until use. Total RNA fractions were used for subsequent experiments only if the A260/280 ratio was in excess than 1.6.

RT-PCR: RT-PCR was performed using the Ready To Go RT-PCR beads (Amersham Biosciences). The reaction was carried out using the two step protocol provided with the kit, with a MyCycler thermal Cycler with gradient module (Bio-Rad, Milan). RT-PCR was carried out mixing 1 μg of total RNA, 0.5 μg of pd (T)12-18, 1 μg of pd (N)6, with RNAse free water. The reaction comprised a reverse transcription step of 30 min (42°C), followed by inactivation of the enzyme at 95°C (5 min). Then 100 pM of specific primers were added and the reactions were cycled for 95°C, 2 min, then 35 cycles of 95°C, 60 s, 47°C, 60 s, 72°C, 60 s, with a final extension at 72°C, 10 min.

Primers used in this study were as follows:

Oxytocin Forward: 5'-TTGCTGTCTGCTCGGCCT-3';
Oxytocin Reverse: 5'-TTTCACCATTTCTGGGGTGG-3' product size 284bp;
ANF Forward: 5'-CGCAGACCTGATGGATTTCA-3';
ANF Reverse: 5'-GCAGCTTAGATGGGATGATCAC-3' product size 486bp;
Beta2 Microglobulin Forward: 5'-CGTCATCCAGCAGA GAATGGAA-3';
Beta2 Microglobulin Reverse: 5'-CCAGATTAA CCACAACCATGCC-3' product size 774bp;
GAPDHForward:5'-AAGGTGAAGGTCGGAGTCAA-3';
GAPDH Reverse: 5'-AAGTGGTCGTTGAGGGCAAT-3'; product size 914bp
Beta Actin Forward: 5'-AAACTGGAACGGTGAAGG TG-3';
Beta Actin Reverse: 5'-TCAAGTTGGGGGACAAAAAG-3'; product size 350bp

Beta2 Microglobulin (B2M) was preferred as housekeeping gene, over GAPDH and beta Actin, for the better linearity of expression in all the experimental conditions.

The identity of PCR products has been confirmed by incubation with the appropriate restriction enzyme and subsequent visualisation of the cleavage products on 2% agarose gel.

Densitometric and statistical analysis: Densitometric analysis of RT-PCR gels has been performed using the software package 1DScan EX (Scanalytics). The expression levels of genes were normalised for the expression of the housekeeping gene, B2M. Statistical

analysis was performed using GraphPad Prism 4.0 Software package. Significance of differences has been assessed by non-parametric Kruskal-Wallis test. Data were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

Immunohistochemistry: Table 1 summarizes immunohistochemical results. In particular, ANF was positive in 42±12% epithelial cells of NB, 40±9% of CIS, 15±4% of N0 and 5±3% of N1 (Fig. 1), statistical analyses showed significant differences during BC progression, as showed in Table 2. By contrast, we found a light positivity for OX only in interstitium in all specimens without significant differences. As discussed in the text, ANF reduces its expression during cancer progression.

CIS : Carcinomas *in Situ*.
N0 : Ductal carcinomas without lymph nodal metastases.
N1 : Ductal carcinomas with lymph nodal metastases.
ANF : Atrial Natriuretic Factor. Original magnification: 40X.

RT-PCR: RT-PCR confirmed the immunohistochemical data; indeed, the expression of ANF mRNA reduced from NB through N0 to N1 (Fig. 2a) statistical analyses of quantification confirmed immunohistochemical results (Fig. 2b). By contrast, OX resulted negative in all examined specimens. Data are represented as mean with the indication of standard deviation, of three replicates. Expression values are represented as O.D./ig of total RNA retrotranscribed, normalised for the expression of the Beta2 Microglobulin. Significance of differences has been assessed by using the Kruskal-Wallis test.

NB : Non-tumoral Breasts.
N0 : Ductal carcinomas without lymph nodal metastases.
N1 : Ductal carcinomas with lymph nodal metastases.
ANF : Atrial Natriuretic Factor.
B2M : Beta2 Microglobulin, as control housekeeping gene.

A number of evidences support an interaction between ANF and oxytocin, paracrine via, in different sites. Haanwinckel *et al*. (1995) showed that oxytocin released from neurohypophysis increases the plasmatic concentration of ANF that, in turn, induces natriuresis and diuresis. Gutkowska *et al*. (1997) showed that oxytocin binds its receptors in the myocardiocytes and induces the release of ANF with a cGMP mediated mechanism, determining a rapid reduction of circulating blood volume. Soares *et al*. (1999) demonstrated an oxytocin-mediated natriuretic action of ANF in renal tubules, with increment of cGMP concentration.

Table 1: Results of semiquantification of immunohistochemical analyses

	NB %	CIS %	N0 %	N1 %
ANF	42±12	40±9	15±4	5±3
OX	5±3	5±3	5±2	4±2

NB: Non-tumoral Breasts; CIS: Carcinomas *in Situ*; N0: Ductal carcinomas without lymph nodal metastases; N1: Ductal carcinomas with lymph nodal metastases; ANF: Atrial Natriuretic Factor; OX: Oxytocin

Table 2: Statistical analyses results showed significant differences between ANF expression, with the exception of NB vs CIS

NB vs CIS	p = 0.55	CIS vs N0	p = 0.01
NB vs N0	p = 0.02	CIS vs N1	p = 0.001
NB vs N1	p = 0.004	N0 vs N1	p = 0.005

NB: Non-tumoral Breasts; CIS: Carcinomas *in Situ*; N0: Ductal carcinomas without lymph nodal metastases; N1: Ductal carcinomas with lymph nodal metastases

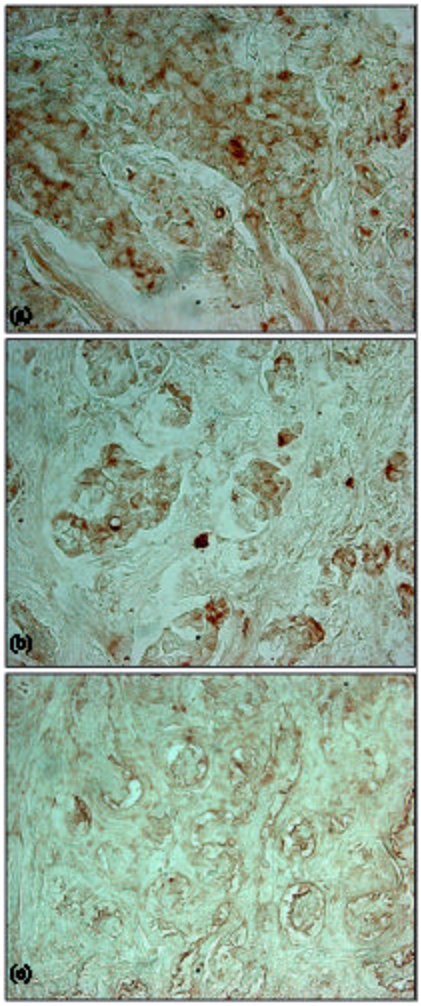

Fig. 1: This panel shows representative results of immunohistochemistry for ANF in a) CIS, b) N0 and c) N1 breast cancer

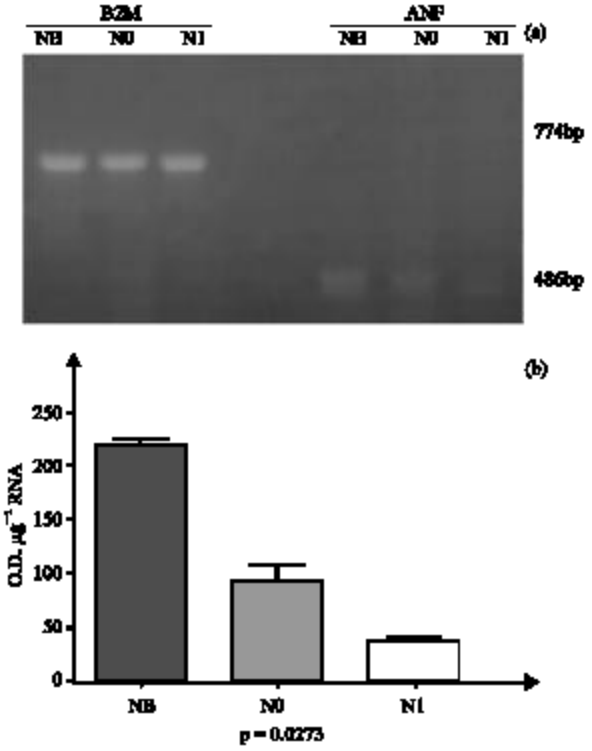

Fig. 2: a) RT-PCR analysis of the expression of the mRNA confirmed ANF reductionfrom NB through N0 to N1 breast cancer. b) Semi-quantitative analyses of RT-PCR results confirmed the reduction of ANF expression, as showed at immunohistochemistry

We recently demonstrated the presence of ANF and OX in the prostate gland; we postulated that both hormones may play a role in the acinary cell function, suggesting its involvement in the composition and fluidity of prostatic fluid (Farina-Lipari *et al*., 2003).

In the present study, we showed that ANF decreases its expression during BC progression. This data may be due with the loss of differentiation of ductal epithelial cells during carcinogenesis. Since, we supposed a paracrine role of ANF in homeostasis of normal breast cells, this hypothesis could be supported from the finding of a reduction of its levels during a de-differentiative process, as cancer.

Finally, since ANF was recently proposed as an anticancer agent (Vesely, 2005), its reduction could be related to BC progression. By contrast, the light immunohistochemical expression of OX and its negativity at RT-PCR confirm that this molecule should not be synthesized in both normal and tumoral gland; indeed, this hormone could be implicated above all during lactation but less during homeostasis of mammal tissue.

In summary, our results encourage to investigate more on presence of ANF and OX in normal and tumoral breast tissue; indeed, a reduction of ANF expression seems to correlate with BC progression. As a consequence, if confirmed in clinical studies on larger series, ANF could become a novel prognostic marker in BC management. By contrast, differently than in rats (Carrera *et al*., 2004), OX should not be involved in breast carcinogenesis, also whether oxytocinase expression should also be considered in these tissues to further confirm these data; at the same time, molecular and proteomic analyses might exclude (or not) a paracrine interaction between these hormones in breast tissue, as already shown in other anatomical districts.

REFERENCES

Atarashi, K., P.J. Mulrow, K. Franco-Saenz, R. Snajdar and J. Rapp, 1984. Inhibition of aldosterone production by an atrial extract. Science, 224: 992-994.

Burnett, J.D., J.P. Granger and T.J. Opgenorth, 1984. Effects of synthetic atrial natriuretic factor on renal function and renin release. Am. J. Physiol., 247: 863-866.

Carrera, M.P., M.J. Ramirez-Exposito, M.T. Valenzuela, M.J. Garcia, M.D. Mayas and J.M. Martinez-Martos, 2004. Serum oxytocinase activity is related to tumor growth parameters in N-methyl nitrosourea induced rat breast cancer. Life Sci., 75: 1369-1377.

Carvajal, J.A., K. Aguan, L.P. Thompson, I.A. Buhimschi and C.P. Weiner, 2001. Natriuretic peptide-induced relaxation of myometrium from the pregnant guinea pig is not mediated by guanilate cyclase activation. J. Pharmacol. Exp. Ther., 297: 181-188.

Currie, M.G., D.M. Geller, B.R. Cole, J.G. Boyland, W.Y. Sheng, S.W. Homberg and P. Needleman, 1983. Bioactive cardiac substances: potent vasorelaxant activity in mammalian atria. Science, 221: 71-73.

Dickson, R.B., 2000. J. Russo Biochemical Control of Breast Development. In: J.R. Harris, M.E. Lippman, M. Morrow (Eds.). Disease of the breast. 2nd Edn. Philadelphia. Lippincott Williams and Wilkins, pp: 15.

Egarter, C.H. and P. Husslein, 1992. Biochemistry of myometrial contractility. Baillieres Clin. Obstet. Gynaecol., 6: 755-69.

Elledge, R.M., 2000. S.A.W. Fuqua, Estrogen and Progesteron Receptors. In: J.R. Harris, M.E. Lippman, M. Morrow (Eds.). Disease of the breast. 2nd Edn. Philadelphia. Lippincott Williams and Wilkins, pp: 471.

Farina-Lipari, E., D. Lipari, M. Bellafiore, R. Anzalone, F. Cappello and B. Valentino, 2003 Presence of atrial natriuretic factor in normal and hyperplastic human prostate and its relationship with oxytocin localisation. Eur. J. Histochem, 47: 133-138.

Favaretto, A.L. and G.O. Ballejo, W.I. Albuquerque-Araujo, J. Gutkowska, J. Antunes-Rodrigues and S.M. McCann, 1997. Oxytocin releases atrial natriuretic peptide from atria in vitro that exerts negative inotropic and chronotropic action. Peptides, 18: 1377-1381.

Gutkowska, J., M. Jankowski, C. Lambert, S. Mukaddam-Daher, H.H. Zingo and S. McCann, 1997. Oxytocin releases atrial naytriuretic peptide by combining with oxytocin receptors in the heart. Proc. Natl. Acad. Sci. USA., 94: 11704-1179.

Haagensen, D.C., 1986. Disease of the breast. 3rd Edn. Philadelphia, WB Saunders.

Haanwinckel, M.A., L.K. Elias, A.L.V. Favaretto, J. Gutkowska and S.M. McCann, 1995. Oxytocin mediates atrial natriuretic peptide release and natriuresis after volume expansion in the rat. Proc. Natl. Acad. Sci. USA., 92: 7902-7906.

McCarty, K.S., L.C. Glaubitz and M. Thienemann, 1983. The Breast: Anatomy and Physiology. In: N.G. Georgiade (Eds.). Aesthetic plastic surgery. Philadelphia: WB Saunders.

McCracken, J.A., E.E. Custer and J.C. Lamsa Luteolysis, 1999. A neuroendocrine-mediated event. Physiol. Rev., 79: 263-323.

O'Brien, W.F., 1995. The role of prostaglandins in labor and delivery. Clin. Perinatol., 22: 973-84.

Pandey, K.N. and M. Orgebin-Crist, 1991. Atrial natriuretic factor in mammalian testis: immunological detection in spermatozoa. Biochem. Biophys. Res. Comm., 180: 437-444.

Samson, W., 1985. Atrial natriuretic factor inhibits dehydration and hemorrhage-induced vasopressin release. Neuroendocrinology, 40: 277-279.

Soares, T.J., T.M. Coimbra, A.R. Martins, A.G.F. Pereira, E.C. Carnio, L.G.S. Branco, W.I.C. Albuquerque-Araujo, G. de Nucci, A.L.V. Favaretto, J. Gutkowska, S.M. McCann and J. Antunes-Rodrigues, 1999. Atrial natriuretic peptide and oxytocin induce natriuresis by release of cGMP. Proc Natl. Acad. Sci. USA., 96: 278-283.

Valentino, B., D. Lipari, G. Peri, A. Gerbino and E.F. Lipari, 2005. ANP-presence and its relationship with the oxytocin in nonlactanting breast of rabbit. Revista Romana de Anatomie Functionala Si Clinica, Macro-Si Microscopica Si de Antropologie, 1: 1-10.

Valentino, B., E.F. Lipari, F. Carini and V. Valenza, 1999. Immunohistochemical localization of Atrial Natriuretic Factor (ANF) in the excretory system of the rabbit parotid gland. Eur. J. Histochem., 43: 241-245.

Vesely, D.L., 2005. Atrial natriuretic peptides: Anticancer agents. J. Investig Med., 53: 360-365.

Vorherr, H., 1974. The breast: Morphology, physiology and lactation. New York; Academic Press.

Vorherr, H., 1980. Breast cancer, epidemiology, endocrinology, biochemistry and pathobiology. Baltimore; Urban and Schwarzenberg.

Wellings, S.R., 1980. A hypothesis of the origin of human breast cancer from the terminal ductal lobular unit. Pathol. Res. Pract., 166: 515-35.

Zingg, H.H., E. Grazzini, C. Breton, A. Larcher, F. Rozen, C. Russo, G. Guillon and B. Mouillac, 1998. Genomic and non-genomic mechanisms of oxytocin receptor regulation. Adv. Exp. Med. Biol., 449: 287-95.