A BRIEF COMMUNICATION

The Source and Secretion of Immunoactive Relaxin in Rat Milk

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The milk of many mammalian species contains hormones and growth factors in addition to nutrients and immuocompetent substances. These factors can be absorbed into the circulation of suckling neonates to exert important effects on metabolism and promote tissue and organ growth. Frequently, there is uncertainty as to whether such substances are gene products of the mammary glands themselves or are produced elsewhere and concentrated from the systemic circulation. The 6 kD polypeptide, relaxin, appears in milk of several mammalian species, including that of the rat, but proof of its source of secretion (corpus luteum vs. mammary gland) is so far lacking. The specific monoclonal anti-rat relaxin antibody MCA1 has previously been utilized successfully to investigate many of relaxin's actions in the rat, including those affecting the development of the mammary apparatus. In this report, MCA1 was utilized to aid in the identification of the source of relaxin in rat milk. Treatment of lactating rats with MCA1 completely neutralized the luteal relaxin circulating in serum but did not decrease the concentration of immunoactive relaxin secreted in milk. Moreover, the antibody did not appear to reach the mammary epithelium. The evidence thus supports the view that in the rat, the relaxin secreted in milk is primarily a product of the mammary glands and not concentrated from the systemic circulation. Exp Biol Med 234:562–565, 2009

Key words: milk-borne relaxin (or lactocrine relaxin); neutralizing antibody; mammary glands; lactating rats

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Introduction

The milk of several mammalian species contains, in addition to nutrients and anti-infectious and immunocompetent substances, so-called "milk-borne trophic factors," including several peptide hormones: growth hormone (GH), insulin, insulin like–growth factor I (IGF-I), epidermal growth factor (EGF), prolactin, luteinizing hormone releasing hormone (LHRH), leptin, and relaxin. These substances can be absorbed into the circulation of neonates to exert important effects on their metabolism and also promote growth and differentiation of several organs and target tissues (1–10).

In many instances it is uncertain whether the milk hormones are gene products of the mammary glands themselves or are produced elsewhere and concentrated from the circulation (11). Relaxin is a case in point, having been detected in human milk (12) and the milk of lactating bitches (13), sows (10), and rats (14). In the human, there is evidence of relaxin gene expression in mammary glands (15), but proof linking gene expression to secretion of relaxin in milk is so far lacking. In the dog (13) and pig (10), there is good evidence that milk-borne relaxin is transmitted to the neonates via suckling, and there is some evidence that a relaxin gene is expressed in the canine mammary glands (13). Although relaxin gene expression has been described in the rat mammary gland by one group (16), another failed to detect its expression in rat mammary tissues (11). Thus the source of milk-borne relaxin in the rat is still uncertain.

The present study enlists the aid of the specific anti-rat relaxin mouse monoclonal antibody MCA1 to identify the source of relaxin in rat milk. MCA1 has been utilized successfully to investigate many of relaxin's actions and targets in the rat (e.g., 17–20). Of particular note was the finding that treatment of pregnant rats with MCA1 prevented lactation in rats and severely restricted nipple growth (19, 20).

Table 1. Immunoactive Relaxin Concentration in Milk of MCA1-Treated and Control Lactating Rats

Group	*N*	Treatment	Sample	Relaxin, ng/ml ± SE
1	5	MCA1 5 mg i.v. ×2d	Breast milk	48.2 ± 2.6
2	5	mIgG 5 mg i.v. ×2d	Breast milk	53.8 ± 8.1
1	5	MCA1 5 mg i.v. ×2d	Pup stomach milk extract	30.0 ± 1.4
2	5	mIgG 5 mg i.v. ×2d	Pup stomach milk extract	32.0 ± 1.4

Accordingly, the objectives of the present experiment were to determine: 1) if treatment of *lactating* rats with MCA1 prevented the appearance of immunoactive relaxin in milk; 2) if MCA1 actually reached any relaxin-producing cells in the rat mammary glands; and 3) if MCA1 neutralized the relaxin secreted by these cells.

Materials and Methods

Antirelaxin Antibody. Mouse monoclonal anti-rat relaxin antibody MCA1, an antibody proven to neutralize the actions of endogenous relaxin on the mammary glands and other tissues in pregnant rats, was prepared in phosphate buffered saline (PBS). Purified mouse IgG (Sigma Chemical Co, St. Louis, MO) in PBS was used as a non-immune control for MCA1. The dose of MCA1 injected was that reported to markedly affect the mammary glands of rats (19, 20).

Experimental Procedure. The protocol was approved by the NYU Institutional Animal Care and Use Committee (IACUC). Ten timed pregnant (mated when 50–55 days old) Sprague-Dawley rats were purchased from Charles River Breeding Laboratories and shipped to our laboratory on the 17th day of gestation. The rats were housed individually in plastic cages, fed Purina Lab Chow and water *ad libitum* in temperature- and humidity-controlled quarters with a 12/12 hr light/dark cycle. On days 1 and 2 after littering, the rats were injected i.v. (tail vein) with either: 1) MCA1, 5 mg in 0.5 ml phosphate buffered saline (5 rats); or 2) purified mouse IgG, 5 mg in 0.5 ml of the same PBS vehicle (5 rats). On day 2 each litter was reduced to an *N* of 5. Twenty-four hr later, under restraint a blood sample (about 1 ml from the tail vein) and a milk sample (from the nipple by syringe) were taken from each of the lactating rats. The mothers and pups were then euthanized by CO_2 inhalation, and one axillary and one thoracic mammary gland was extirpated and fixed in 10% neutral buffered formalin for determination of the presence of antirelaxin antibody by immunohistochemistry. The stomach contents of one pup from each litter were also removed and assayed for relaxin in the RIA after precipitating the milk proteins by acid extraction (0.1N HCl) and neutralizing the extract with 0.1N NaOH (21). Relaxin concentration in serum and milk was determined by radioimmunoassay.

Analytical Methods. The rat relaxin RIA was conducted exactly as described by Sherwood et al (22). Samples of milk and serum were run in triplicate in 100 µl volumes against a synthetic rat relaxin standard. Possible binding of MCA1 to mammary gland cells was evaluated by the Kaplan Comprehensive Cancer Center Experimental Animal and Histopathology Shared Resource using an avidin-biotin complex immunoperoxidase reaction after exposing the tissue sections to biotin-labeled rabbit anti-mouse IgG. Mammary gland tissue sections of rats treated with non-immune mouse IgG served as controls.

In order to estimate the success of the neutralization of circulating (ovarian source) relaxin, the following was considered: the primary antiserum used in the rat relaxin RIA (22) is *a rabbit anti-rat polyclonal antiserum*. After addition of the ^{125}I-labeled rat relaxin tracer to the rat serum samples, the rabbit anti-rat relaxin antiserum is added to bind specifically all relaxin (endogenous plus trace) present in the assay tube. This antigen-antibody complex is then precipitated using a goat-anti-rabbit IgG second antibody. The radioactivity in the precipitate is then determined, and the amount of relaxin originally present in the sample is calculated by the displacement of the ^{125}I-labeled relaxin (tracer = 100% bound in the absence of non-radioactive relaxin). However, the serum of the MCA1-treated rats contained sufficient MCA1 mouse anti-rat antibody to bind about 68% of the ^{125}I-labeled rat relaxin trace prior to addition of the second antibody. As the second antibody was specific for rabbit IgG, it did not precipitate the mouse (MCA1)-bound radioactivity.

Results are presented as mean values ± SEM. Significance of differences was calculated by Student's *t* test with significance of differences set at $P = 0.05$.

Results and Discussion

Relaxin immunoactivity was detected in the milk of lactating rats and in the stomachs of suckling pups. The relaxin concentration in the breast milk and ingested milk was similar in the rats treated with MCA1 and in the controls treated with the non-immune mIgG (Table 1).

Avidin-biotin immuno-peroxidase staining did not reveal mouse IgG in any of the cells of the mammary glands of the MCA1-treated or mIgG-treated lactating rats (data not shown). Light immunostaining was occasionally observed in blood vessels within and adjacent to the mammary glands of MCA1-treated rats, consistent with the presence of the antibody in the circulation. Therefore, it appears that the MCA1 antibody does not reach the mammary epithelium, in harmony with the previous report that circulating MCA1 does not prevent relaxin from binding to sites in the mammary epithelium (23). The cellular location of specific LGR7 relaxin receptors in rat

Table 2. Antirelaxin Activity in Serum of MCA1-Treated Lactating Rats*

			Specific ^{125}I-Relaxin binding	
N	Treatment	Sample	Observed in RIA %	Bound by serum %
5	MCA1 5 mg i.v. ×2da	Serum (100 μl)	32.2 ± 2.1	67.8[a]
5	mIgG 5 mg i.v. ×2da	Serum (100 μl)	94.7 ± 5.9	5.3[b]

[a] ^{125}I-Relaxin bound by rat serum before precipitation with second antibody ($P < 0.001$).
[b] ^{125}I-Relaxin bound by rat serum before precipitation with second antibody (N.S.).
* A rabbit anti-rat relaxin polyclonal antiserum is the primary antibody used in the RIA. This is then precipitated using a goat-antirabbit IgG second antibody. The serum of the MCA1-treated rats contained sufficient mouse anti-rat relaxin antibody to bind about 68% of the ^{125}I-labeled rat relaxin trace prior to addition of the second antibody. However, as the second antibody was specific for rabbit IgG, it did not precipitate mouse IgG (MCA1)-bound radioactivity.

mammary glands has not yet been described, but in LGR7 knockout mice, only the nipples appear to be affected; the nipples fail to develop, but glandular development and milk production are not markedly affected (24). Similarly, relaxin deficient (KO) mice produce milk, but their nipples fail to develop (25). Thus nipple maturation but not mammary gland development and milk production appear to depend upon relaxin and its receptors. A similar situation may exist in the rat, as MCA1 treatment resulted in failure of the nipples to mature, but mammary glands were much less affected by neutralization of relaxin (20).

In view of the foregoing, further studies are planned to determine the site(s) of relaxin gene expression in the rat mammary gland, incorporating both immuno-cytochemical and real-time polymerase chain reaction technologies.

As noted above under "Materials and Methods," MCA1 was detected in a serum concentration sufficient to bind 68% of the iodine labeled relaxin in the RIA, indicating antibody excess and therefore suggesting that all circulating ovarian relaxin had been successfully neutralized (Table 2). As MCA1 treatment did not affect the concentration of relaxin in the milk, the latter could not have been derived from the circulation.

The foregoing evidence thus suggests that treatment of rats with MCA1 may prevent *ovarian* relaxin from reaching the mammary glands via the circulation but does not interfere with relaxin secretion by the mammary glands. We therefore conclude that the relaxin immuoactivity detected in rat milk is primarily a product of the mammary glands and not derived from circulating ovarian relaxin.

While it may be argued that Chen et al (11) did not find evidence of relaxin gene expression in mammary glands of rats on the tenth or twenty-first day of lactation, our study was carried out on the first three days of lactation, when relaxin concentrations were highest in milk. Also, Gunnersen et al (16) did find expression of the relaxin gene in rat mammary glands in their studies.

Finally, the effects of milk-borne relaxin on development of the suckling rat pups have not yet been investigated. However, relaxin as a milk constituent has been reported to play an important role in uterine and cervical development in suckling piglets (26) and serves as the exemplary hormone for the *lactocrine hypothesis* (27). Indeed, when the distribution of relaxin receptors is considered, one may further speculate that, in addition to effects on the reproductive system, milk-borne relaxin conceivably could play a significant role in early development of the cardiovascular, genitourinary, and nervous systems (28).

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1. Buts JP. Bioactive factors in milk. Arch Pediatr 5:298–306, 1998.
2. Burrin DG, Stoll B. Key nutrients and growth factors for the neonatal gastrointestinal tract. Clin Perinatol 29:65–96, 2002.
3. Koldovsky O. The potential physiological significance of milk-borne hormonally active substances for the neonate. J Mammary Gland Biol Neoplasia 1:317–323, 1996.
4. Dvorak B, Williams CS, McWilliam DL, Shinohara H, Dominguez JA, McCuskey RS. Milk-borne insulin with trypsin inhibitor in milk induces pancreatic amylase development at the onset of weaning in rats. Pediatr Gastroenterol Nutr 30:515–521, 2000.
5. Huo YJ, Wang T, Xu RJ, Macdonald S, Liu G, Shi F. Dietary insulin affects leucine aminopeptidase, growth hormone, insulin-like growth factor I and insulin receptors in the intestinal mucosa of neonatal pigs. Biol Neonate 89:265–273, 2006.
6. Philipps AF, Koldovsky O. Milk-borne epidermal growth factor modulates intestinal transforming growth factor-alpha levels in neonatal rats. Pediatr Res 47:194–200, 2000.
7. Ellis LA, Mastro AM, Picciano MF. Milk-borne prolactin and neonatal development. J Mammary Gland Biol Neoplasia 1:259–269, 1996.
8. Gama P, Alvares EP. Localization of luteinizing-hormone releasing hormone binding sites in the gastric mucosa of suckling rats. Anat Rec 264:43–50, 2001.
9. Miralles O, Sánchez J, Palou A, Picó C. A physiological role of breast milk leptin in body weight control in developing infants. Obesity (Silver Spring) 14:1371–1377, 2006.
10. Yan W, Wiley AA, Bathgate RA, Frankshun AL, Lasano S, Crean BD, Steinetz BG, Bagnell CA, Bartol FF. Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. Endocrinology 147: 4303–4310, 2006.
11. Chen A, Laskar-Levy O, Koch Y. Selective expression of neuropeptides in the rat mammary gland: somatostatin gene is expressed during lactation. Endocrinology 140:5915–5921, 1999.
12. Eddie LW, Sutton B, Fitzgerald S, Bell RJ, Johnston PD, Tregear GW.

Relaxin in paired samples of serum and milk from women after term and preterm delivery. Am J Obstet Gynecol 161:970–973, 1989.
13. Goldsmith LT, Lust G, Steinetz BG. Transmission of relaxin from lactating bitches to their offspring via suckling. Biol Reprod 50:258–265, 1994.
14. Steinetz BG, Sherwood OD, Lasano S, Horton L, Bosland MC. Immuno-neutralization of circulating relaxin does not alter the breast cancer-protective action of parity in MNU-treated rats. J Exp Ther Oncol 4:59–68, 2004.
15. Tashima LS, Mazoujian G, Bryant-Greenwood GD. Human relaxins in normal, benign and neoplastic breast tissue. J Mol Endocrinol 12:351–364, 1994.
16. Gunnersen JM, Crawford RJ, Tregear GW. Expression of the relaxin gene in rat tissues. Mol Cell Endocrinol 110:55–64, 1995.
17. Hwang JJ, Shanks RD, Sherwood OD. Monoclonal antibodies specific for rat relaxin. IV. Passive immunization with monoclonal antibodies during the antepartum period reduces cervical growth and extensibility, disrupts birth, and reduces pup survival in intact rats. Endocrinology 125:260–266, 1989.
18. Lee AB, Hwang JJ, Haab LM, Fields PA, Sherwood OD. Monoclonal antibodies specific for rat relaxin. VI. Passive immunization with monoclonal antibodies throughout the second half of pregnancy disrupts histological changes associated with cervical softening at parturition in rats. Endocrinology 130:2386–2391, 1992.
19. Hwang JJ, Lee AB, Fields PA, Haab LM, Mojonnier LE, Sherwood OD. Monoclonal antibodies specific for rat relaxin. V. Passive immunization with monoclonal antibodies throughout the second half of pregnancy disrupts development of the mammary apparatus and, hence, lactational performance in rats. Endocrinology 129(6):3034–3042, 1991.
20. Kuenzi MJ, Sherwood OD. Monoclonal antibodies specific for rat relaxin. VII. Passive immunization with monoclonal antibodies throughout the second half of pregnancy prevents development of normal mammary nipple morphology and function in rats. Endocrinology 131:1841–1847, 1993.
21. Steinetz BG, Gordon T, Lasano S, Horton L, Ng SP, Zelikoff JT, Nadas A, Bosland MC. The parity-related protection against breast cancer is compromised by cigarette smoke during rat pregnancy: observations on tumorigenesis and immunological defenses of the neonate. Carcinogenesis 27:1146–1152, 2006.
22. Sherwood OD, Crnekovic VE, Gordon WL Rutherford JE. Radioimmunoassay of relaxin plasma levels throughout pregnancy and during parturition in the rat. Endocrinology 107:691–698, 1981.
23. Kuenzi MJ, Sherwood OD. Immunohistochemical localization of specific relaxin-binding cells in the cervix, mammary glands, and nipples of pregnant rats. Endocrinology 136:1367–1373, 1995.
24. Krajnc-Franken MAM, van Disseldorp AJM, Koenders JE, Mosselman S, van Duin M, Gossen JA. Impaired nipple development and parturition in LGR7 knockout mice. Mol Cell Biol 24:687–694, 2004.
25. Zhao L, Roche PJ, Gunnersen JM, Hammond VE, Tregear GW, Wintour EM, Beck F. Mice without a functional relaxin gene are unable to deliver milk to their pups. Endocrinology 140:445–453, 1999.
26. Yan W, Wiley AA, Bathgate RA, Frankshun AL, Lasano S, Crean BD, Steinetz BG, Bagnell CA, Bartol FF. Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. Endocrinology 147: 4303–4310, 2006.
27. Bartol FF, Wiley AA, Bagnell CA Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. Reprod Domest Anim 43(Suppl 2):273–279, 2008.
28. van der Westhuizen ET, Halls ML, Samuel CS, Bathgate RA, Unemori EN, Sutton SW, Summers RJ. Relaxin family peptide receptors–from orphans to therapeutic targets. Drug Discov Today 3:640–651, 2008.