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FOOD & FUNCTION

Breast milk contains relevant neurotrophic factors and cytokines for enteric nervous system development

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Breast-feeding plays an important role for the development of the newborn. Non-breast fed premature born infants show a significantly higher risk of developing diseases like infantile diarrhoea and necrotizing enterocolitis. In this study, the content of neurotrophic factors and cytokines, which might influence the postnatal development of the enteric nervous system (ENS), was determined in human breast milk. Glial cell-line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) as well as a panel of cytokines were analyzed using single factor or multiplex ELISA. In order to link their presence in milk with possible effects on the development of the ENS, rat myenteric neurons were cultured in protein extracts from breast milk. Neurite outgrowth, neuron survival and nestin expression in glial cells were measured. Growth factors and cytokines were found in all breast milk samples at varying concentrations. It could be demonstrated that protein extracts of breast milk increased the amount of surviving enteric neurones as well as neurite outgrowth. Additionally it was shown, that the number of nestin and S100-expressing glial cells increased significantly after incubating in breast milk protein extracts. The data suggest that milk-born proteins support the development of the enteric nervous system.

Keywords:
Breast milk / CNTF / cytokines / GDNF / Myenteric plexus

Breast-feeding is important for the development of the newborn infant. Since the newborn intestine is immature, breast milk contains compounds e.g. a broad range of cytokines like TGF-β [1], interleukins like tumor necrosis factor (TNF)-α or RANTES [2] that sustain gut maturation and immunological development of the infant. This results in a reduced risk of developing pathological conditions such as infantile diarrhoea [3] or necrotizing enterocolitis (NEC) [4], or which are based on dysregulated immunity like ulcerative colitis or Crohn's disease [5].

Since the newborn enteric nervous system is not yet fully developed at birth [6, 7] an appropriate supply of neurotrophic factors is needed during the first postnatal weeks. GDNF (glial-cell-line-derived-neurotrophic-factor), a member of the TGF-β-superfamily [8] is a very important growth factor for the development and more importantly neuronal survival of the enteric nervous system (ENS), since the lack of GDNF leads to a nearly complete loss of all enteric neurons [9]. In vitro studies of dissociated myenteric plexus from newborn rats showed that GDNF improved

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Abbreviations: CNTF, ciliary neurotrophic factor; **ENS,** enteric nervous system; **GDNF,** glial cell-line-derived neurotrophic factor; **MIP,** macrophage inflammatory protein; **NEC,** necrotizing enterocolitis; **TNF,** tumor necrosis factor

neurite outgrowth and survival [10]. CNTF (ciliary neurotrophic factor) exerts similar effects [11]. So far, neither GDNF nor CNTF have been demonstrated in human breast milk. We therefore investigated whether these factors and relevant cytokines are present in human breast milk and if so, whether a protein extract of breast milk can influence the survival and neurite outgrowth of isolated rat myenteric neurons.

Nearly, 164 human breast milk samples delivered at subsequent time points from 14 individual mothers were analysed concerning the amount of CNTF, GDNF and TGF-β using ELISA or bioassays [12]. In some cases also colostrum could be obtained. Additionally, a multiplex assay was performed to analyze and quantify cytokine content. All samples were treated with standardized protocols, starting with the assessment of the sample, storage, transportation and analysis, to avoid a potential bias due to changing conditions.

9 individual samples from 3 different mothers with different GDNF, and/or CNTF content were used for the cell culture experiments. Proteins were extracted by precipitation using a 2D Clean-up Kit (GE Healthcare) and diluted in Neurobasal-A-Medium (Gibco).

Myenteric plexus was isolated from small intestine of newborn rats [13]. Briefly, after decapitation, the small intestine was dissected, the muscle layer removed and incubated in calcium- and magnesium-free Hanks balanced salt solution (HBSS, Gibco Life Technologies) containing collagenase II (CLSII, Worthington, 1 mg/mL), trypsin-chymotrypsin-inhibitor (Sigma-Aldrich Chemie GmbH) and DNAse (Roche Diagnostics GmbH) for 2 h at 37°C. Isolated plexus was collected and stored in MEM-HEPES on ice. Myenteric plexus tissue was subjected to a second digestion in Accutase (PAA) for 10 min at 37°C. After trituration using a 27-gauge needle, the cells were centrifuged at 800 rpm. After diluting the pellet with differentiation medium, viable cells were counted and 10 000 cells per well were seeded on poly-L-lysine (70–150 kDa, 50 μg/mL)-coated coverslips. After 45 min of incubation 420 μL medium was added to the wells. Differentiation medium (96% Neurobasal-A-Medium, 1% bovine serum albumin-BSA (Sigma-Aldrich), 1% penicillin/streptomycin/neomycin, 2% B27+retinoic acid (Sigma-Aldrich), 0.1% β-mercaptoethanol (50 mM, Gibco), 0.25% L-glutamine (200 mM, Sigma-Aldrich)) and differentiation medium enriched with GDNF (10 ng/mL) were provided as the control media. The effects of the human breast milk samples were tested by adding the extracted proteins to the differentiation medium at a concentration of 500 ng protein/mL. The cells were grown for 48 h, fixed with 4% formaldehyde (AppliChem), immunostained for the neuronal marker tubulin E7 (mouse-ßIII-tubulin, Hybridoma tissue bank) and visualized with a fluorescence secondary antibody (ALEXA goat-α-mouse 488). Neuronal numbers and neurite outgrowth were measured using the image analysis software ImageJ, including the plug-in NeuronJ. For each condition at least 100 randomly chosen neurons were measured. Additionally, long-term cultures (4 days in vitro) were performed to assess the amount of the nestin-positive cells within the glial population. Enteric glial cells were identified by S 100 stainings [15]. All experiments were performed in triplicates. Statistical analysis of neurite outgrowth was performed using a Wilcoxon rank test. All sample effects were compared with the control and statistical significant difference was accepted at *p*-values smaller than 5%.

A total of 28 samples were analyzed for their concentrations of the factors GDNF, CNTF and TGF-β. No time-dependence was observed of the TGF-β and CNTF concentrations, while the GDNF amount usually decreased with increasing postpartal period (ranging from 2 to 18 pg/mL for GDNF, 10 to 50 ng/mL for CNTF and 2 to 1 ng/mL for TGFß), showing highest concentrations in the colostrum samples. There were samples that contained all neurotrophic factors measured, while others contained TGF-β and GDNF or only TGF-β alone.

Besides the trophic factors described above, a pattern of cytokines was detected: ENA78, GRO-α, Leptin, MCP-1, macrophage inflammatory protein (MIP-1-α), MIP-1-β, RANTES and members of the interleukin family (IL-17, IL-1a, IL-1b, IL-6, IL-7 and IL-8). The concentration of most of the cytokines clearly increased with increasing age of the neonate (ENA78, GRO-α, MIP-1-α, MIP-1-β, IL-17, IL-1α, IL-6, IL-7). Only for RANTES, the concentration was inversely related to the age of the newborn.

Evaluation of neuronal growth was performed on individual neurones (Fig. 1). The difference in neurite outgrowth between medium enriched with breast milk protein extracts and the control medium was evaluated by measuring the length of 100 neurones per experiment. The average neurite outgrowth of neurons was 55.34 μm ± 26.33 μm in differentiation medium alone and 83.08 μm ± 43.83 μm supplemented with GDNF. Every breast milk sample promoted the cell's neurite outgrowth up to 178.19% (BM 12/1) when compared with differentiation medium alone. Neuronal survival was also increased up to 147.92% under the influence of breast milk (BM 13/11) and the addition of GDNF led to an enhancement of 146.25%.

To obtain a more realistic impression of the total breast milk effect upon enteric neurones, total neurite outgrowth was calculated by multiplying the average neurite outgrowth with the average number of surviving neurones. This combination results in a benefit of up to 237.20% ± 38.18 in the breast milk samples and 219.57% ± 35.34% in the GDNF-control, (Fig. 1C) when compared with differentiation medium alone. Cultures fed with breast milk presented a much higher percentage of nestin-immunoreactive cells (77.09% ± 9.40%) (Fig. 2B and C) while, among the cells cultured under control conditions only 46.09% ± 20.50% were positive for nestin (Fig. 2A and C).

In this study, breast milk promoted neurite outgrowth and survival of dissociated myenteric neurons from postnatal rats, by preventing apoptosis. During the postnatal period, the enteric nervous system undergoes significant changes [7, 14]. With this ''neuroplastic strategy'', the

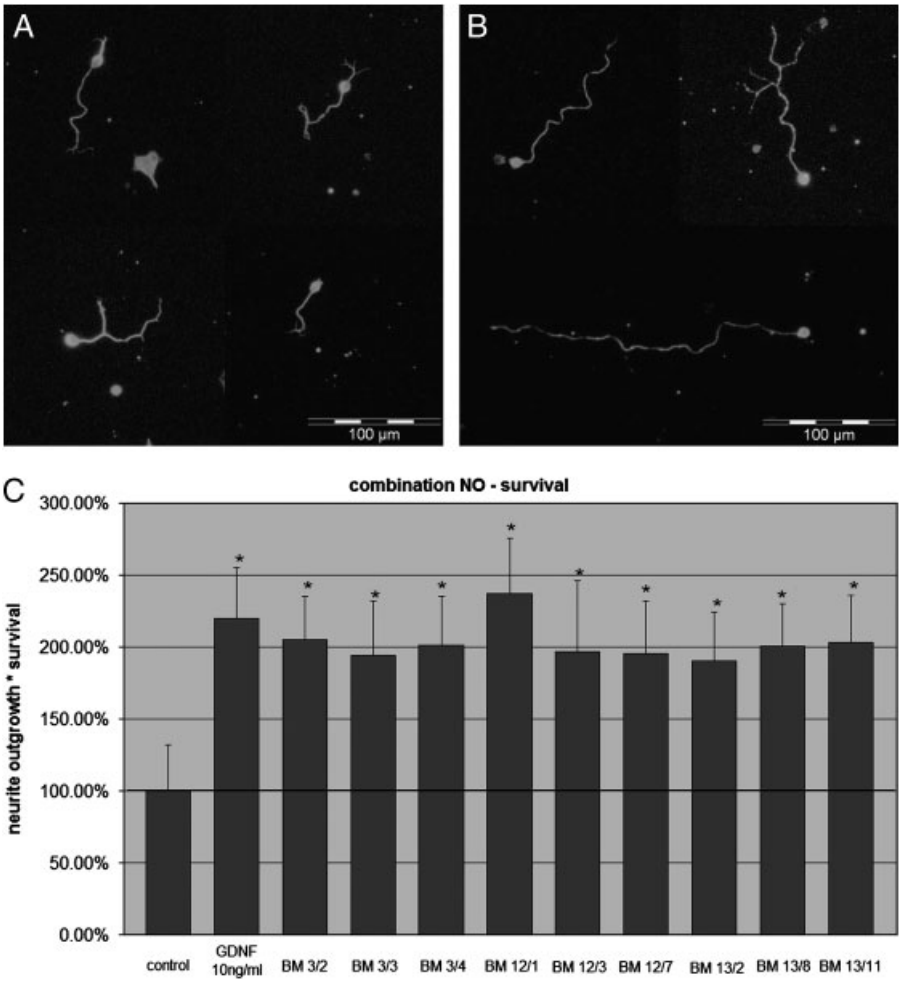

Figure 1. Dissociated myenteric plexus cells cultured for 48 h on poly-L-lysine-coated cover slips in differentiation medium (A) and breast milk medium (B). Single neurons immunostained against tubulin were analysed concerning their neurite outgrowth. (C) The combination of neurite outgrowth and survival analysis showed significant promoting effects of breast milk samples upon myenteric plexus cells in vitro.

enteric nervous system responds to new situations inherent to the postnatal period such as changes of gut motility, microenvironment, dietary habits and microflora [16]. Thus, the bioavailability of neurotrophic factors and cytokines on certain points of time is essential to ensure the optimal rearrangement of the enteric nervous system, and in consequence the development of the newborn gut. Breast milk as the first food a newborn receives, is ideally placed to provide the infant with these essential factors.

Although both GDNF and CNTF immunoreactive material found in the breast milk samples are very likely to correspond to the respective neurotrophins, further investigations will be necessary to proof their identity. Moreover, it has to be investigated whether the individual variation of trophic factor content is related to specific conditions, such as ethnic background, diet, smoking or diseases of the mother, etc.

How much of the factors will be resorbed by the intestinal wall is unclear. However, the proteolytic fate of milk-born proteins is low since the conditions for effective proteolysis are not fully developed in the newborn [17]. In addition, the mucosal barrier of the human newborn gut allows macromolecular transport [14, 18], creating the possibility that milk-born factors can cross the epithelial barrier and come in close contact with parts of the enteric nervous system [18–20]. GDNF is a very important factor for the development of the ENS [6, 9]. Another neurotrophic factor is CNTF, which shows similar effects as GDNF [11]. Our in vitro results confirm earlier observations that human recombinant GDNF promotes neurite outgrowth as well as neuronal survival in dissociated myenteric plexus [10]. Protein extracts increased neurite outgrowth and neuronal survival to a level comparable with GDNF (10 ng/ml), although the levels of GDNF are much lower in the milk samples.

The concentration of the growth factors in breast milk samples could not be correlated directly to the values obtained in neurite outgrowth, neuronal survival or presence of non-neurite-bearing neurons. This lead to the conclusion, that the effect cannot be attributed to a specific factor or a specific combination or concentration of neurotrophic factors. Besides influencing growth and survival of myenteric neurons, breast milk also affected the enteric glial fate. The enteric glia responded to the treatment in expressing nestin in a higher number of glial cells. The number of nestin-positive cells was significantly increased after the

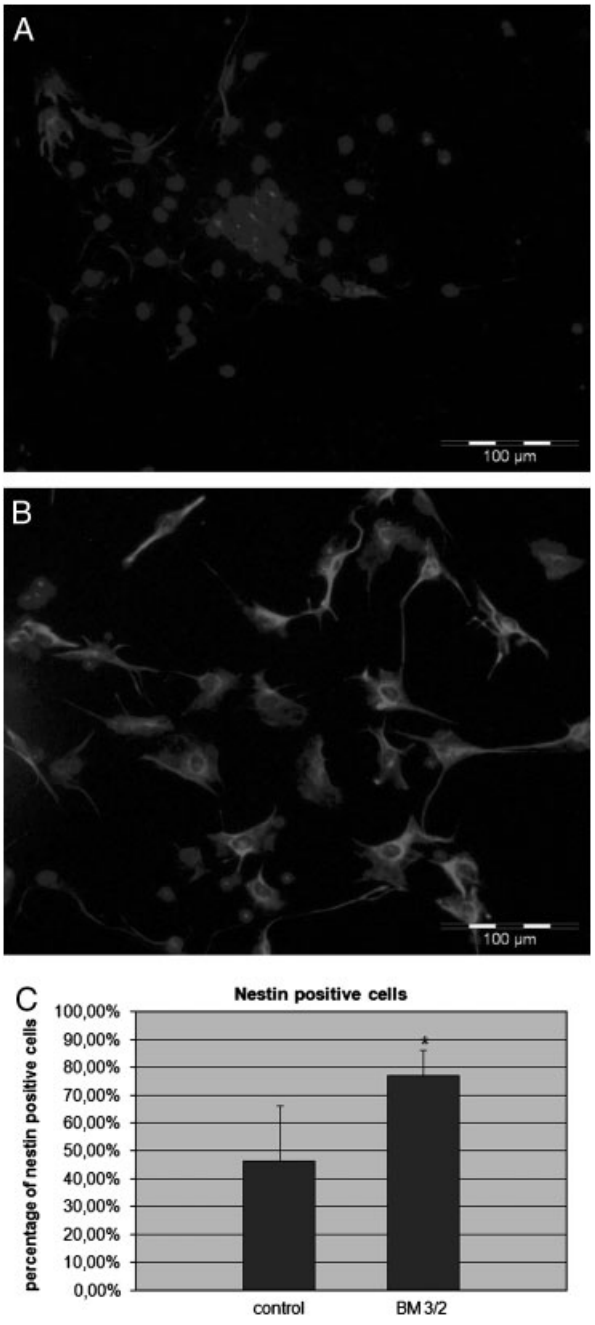

Figure 2. (A) and (B) Myenteric plexus ganglia were cultured in control (A) and breast milk supplemented (B) media and immunostained for Nestin (somatic staining) and DAPI (nuclear staining). (C) Nestin expression is induced by adding breast milk samples to the culture media. The percentage of Nestin-positive cells among the total number of cells was more prominent under the influence of breast milk.

exposure to breast milk proteins. Owing to the fact that nestin is expressed by neural stem cells [21, 22], these findings illustrate that breast milk – possibly by means of neurotrophic factors e.g. GDNF [23] – is able to differentiate the neuroglial stem cells into neurons.

Breast milk has a protective character to prevent from diseases like NEC [4] or infantile diarrhoea [3]. NEC can be described as an ischemic bowel disease, which is characterised by acute inflammation and necrosis of the gut wall. Though, the exact pathogenesis is still unknown, a few studies showed that there is a correlation of NEC and an upregulated expression of proinflammatory cytokines [24] leading to the suggestion that anti-inflammatory cytokines or factors could reduce the risk of developing NEC. Several studies suggest that anti-inflammatory milk-born components could help to protect from these diseases [25, 26]. Breast milk has been demonstrated to contain a broad range of different cytokines, like tumor necrosis factor (TNF)-α, Interferon (IFN)-γ, RANTES, monocyte chemotactic protein (MCP)-1, MIP-1-α and a lot of members of the interleukin family (IL-1, IL-6, IL-8, IL-10) [2]. These data are consistent with our findings. Additionally, we found ENA78, GRO-α, Leptin, IL-7 and IL-17. Even though many cytokines have been identified, a change in cytokine concentration with increasing age has not yet been described. The physiological significance of the increasing with age concentration of several of the cytokines remains to be elucidated. Our results suggest that breast milk is capable of promoting neuronal differentiation, neurite outgrowth and neuronal survival of dissociated myenteric plexus from rats. The mix of cytokines and growth factors present in breast milk are suggested to play a role in the observed effects. Moreover, the link between the enteric nervous system and milk born cytokines and growth factors provide a new point of view in further elucidating the ethiopathogenesis of diseases like NEC, Crohn's disease or infantile diarrhoea.

The authors have declared no conflict of interest.

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