Growth Hormone & IGF Research 22 (2012) 59–63

Contents lists available at SciVerse ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

Visualization of immunoreactive growth hormone in cultured peripheral bovine lymphocytes

Sabine Klein, Nahid Parvizi *

Department of Functional Genomics and Bioregulation, Institute of Farm Animal Genetics Mariensee, FLI, Höltystr. 10, 31535 Neustadt, Germany

ARTICLE INFO

Article history:
Received 3 August 2011
received in revised form 10 January 2012
accepted 20 January 2012
Available online 15 February 2012

Keywords:
Growth hormone
Intracellular localization
Con-focal microscopy
Lymphocyte

ABSTRACT

Growth hormone (GH) has been shown to be released by immune cells in vitro. Thus, the intracellular confinement of GH immunoreactivity was investigated in cultured bovine lymphocytes using con-focal microscopy. Peripheral blood lymphocytes from cows in early pregnancy (10–20 days post insemination; pi) or during mid-pregnancy (day 110–140 pi) were harvested and cultured for 48 h in presence of phytohemagglutinin-M (PHA-M) or served as controls. Thereafter, immunocytochemistry was conducted using a homologous GH-antibody. Double staining (GH-antibody and directly DYE 549 labeled CD3-antibody) was performed to classify the cells. Con-focal laser scanning was applied verifying the immunofluorescence labeling. Interestingly, the presence of GH immunoreactivity in the cytoplasm, which indicates GH synthesis, was restricted to small cells. Whereas, few T-like cells revealed surface bound GH. Lowest immunoreactivity, concerning the number of the total labeled cells as well as the intensity of labeling was recorded in early pregnancy. Stimulation with PHA-M enhanced total labeled cells in early pregnancy. In contrast, PHA-M had no such effects in mid-pregnancy. The results confirm the specific regulation of synthesis of lymphocytic GH during pregnancy in the cow. The identification of cells producing GH and the elucidation of the mechanisms underlying the expression of GH in larger number of cells during mid-pregnancy than in the early pregnancy need further investigations.

1. Introduction

Bidirectional communication between the immune and the neuroendocrine system is mediated by a common set of signaling molecules including hormones, cytokines, and receptors of these mediators [1–3].

Growth hormone (GH) is one of the first classical hormones, which was identified in lymphocytes [4]. Lymphocytes not only produce GH [5–8] but also express GH receptor [5–9]. The lymphocytic GH is apparently similar to its pituitary counterpart in the human [10] and in the pig [8]. Growth hormone is involved in the development and regulation of immune system [11,12]. It modulates the migration of developing T cells [13] and improves the leukocyte's function in ovariectomized old rats [14]. Further, GH enhances the function of thymus in HIV-1-infected men [15]. Chung et al. [16] reported a negative regulation of calcium binding pro-inflammatory S100 proteins by GH in human white blood cells. At gene level, a close relationship is found between GH-gene expression and the activity of the hemolytic complements (CH50 and AH50) in the pig [17]. Taken all this together there is a close relationship between immune system and GH.

We previously demonstrated adrenocorticotropin (ACTH) synthesis in bovine lymphocytes, which is highlighted during the pregnancy [18]. We also have shown that bovine lymphocytes harvested from cyclic as well as pregnant cows secret GH when cultured in vitro [8,19]. Thus, the present study aimed to specify to which extent the cellular immunoreactive GH can be visualized in cultured bovine lymphocytes. To achieve this aim, lymphocytes from cows in early (10 to 20 days post insemination; pi) and mid-pregnancy (110 to 140 days pi) were cultured with or without phytohemagglutinin-M (PHA-M). The types and number of GH-labeled lymphocytes, as well as the intensity and distribution of labeling within the cells were analyzed.

2. Materials and methods

2.1. Preparation of lymphocytes

A total of 12 pregnant Holstein Frisian cows (HF, 5.4 ± 1.2 years old; in their 1–3 lactation) from the research farm of the Institute of Farm Animal Genetics were included in the experiment. Animals were divided into two groups according to the stage of pregnancy. Group one was composed of four cows during early pregnancy (10 to 20 days pi). Group two was comprised of 8 cows in mid-pregnancy (110–140 days pi). The peripheral lymphocytes were separated as described previously [18]. A minimum of 250 ml blood was

* Corresponding author at: Department of Functional Genomics and Bioregulation, Institute of Farm Animal Genetics, FLI, Höltystrasse 10, Mariensee, D-31535, Neustadt a. Rbg., Germany. Fax: +49 5034 871 247.
E-mail address: nahid.parvizi@fli.bund.de (N. Parvizi).

1096-6374/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ghir.2012.01.001

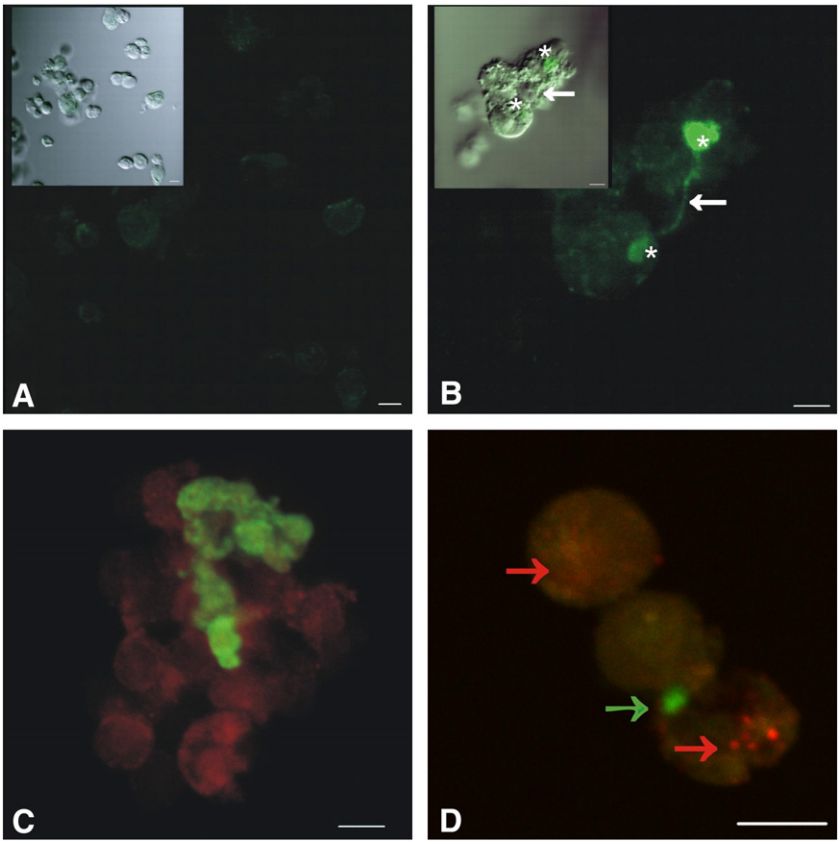

Fig. 1. Bovine lymphocytes labeled with anti-bovine GH raised in rabbits and visualized with anti-rabbit antibody conjugated to FITC: A) The FITC channel of a control sample without primary GH antibody. The inset shows the projection overlay of differential interference contrast and fluorescence channel of this region. For more detail see the text. B) FITC-channel projection showing two GH positive lymphocytes (*). The two GH-labeled cells are connected via a tread-like structure (→), which is also GH positive and could indicate cell to cell communication. The inset demonstrates the arrangement of different lymphocytes as overlay with the differential interference contrast. C) Projection overlay of a region with small cells with GH immunoreactivity (green) attached to a larger cluster of T cells identified by CD3 immunoreactivity (red). This arrangement was characteristic for lymphocytes stimulated with PHA-M. The lack of yellow color suggests the absence of GH in these CD3 cells. D) A 2-channel projection overlay of a sample with one small GH positive cell (green arrow) between 3 large T cells. Two of the T cells are positive for the CD3 label (red arrows). All scales 5 μm. All cells are PHA-M stimulated.

withdrawn from the jugular vein between 8 and 9 o' clock a.m. The EDTA mixed blood was centrifuged. Thereafter, the buffy coat was re-suspended in Hanks balanced salt solution (HBSS, H2387, SIGMA, Taufkirchen, Germany). Lymphocytes were separated with lymphodex (H9L5049, Inno-Train, Kronberg, Germany), transferred to fresh tubes and washed 3 times with HBSS. Remaining erythrocytes were lysed for 10 s in distilled water. Cells (1×10^6 per ml) were transferred to the culture medium consisting of 1:1 (v:v) RPMI-1640 (SIGMA, Germany) and HBSS. This culture medium was supplemented with 1% normal calf serum (NCS, N4762, SIGMA) and 1% antibiotic and antimycotic solution (A5655, Sigma). The cells were seeded in four-well plates (Nunc Brand Products, Darmstadt, Germany) and incubated at 37 °C and in 5% CO_2 and 95% air. After 24 h of adaptation, medium was changed for all cultures and 25 μg/ml PHA-M (from *Phaseolus vulgaricus* L8902, SIGMA) was added to half of the cell cultures. After 48 h of incubation cells were washed once in PBS, and fixed in 3.7% formaldehyde (1.04002, Merck Darmstadt, Germany) for 15 min and dehydrated in ascending concentrations of methanol (30%, 50% and 100%) solution. Samples of 30×10^6cells were stored at −20 C for immunocytochemistry. A CD3 antibody was used to verify T cells (see Immunocytochemistry).

Viability of the cells was assessed in fresh cultures and after the incubation period using trypan blue exclusion test. This test revealed seeding of more than 96% living cells in fresh cultures. After three days of culture, the proportion of living cells in controls varied between 82 and 94%, whereas in PHA-M stimulated cultures 74 to 85% of cells proved alive.

2.2. Immunocytochemistry

Samples of 30×10^6cells each were rehydrated in 50, 70 and 100% PBS, and washed once in 0.02 M PBS. To block nonspecific binding, the rehydrated cells were incubated in PBS containing 5% normal goat serum (NGS) (DAKO, X0907, Glostrup, Denmark), 2% bovine serum albumin, and 0.2% Triton X100 for 30 min. Thereafter, the cells were transferred to a PBS buffer containing anti-bovine growth hormone antibody raised in rabbit (AbD Serotec, 4750–0956, diluted 1:4000, Morphosys, Oxford, UK,) or the anti-human CD3 antibody raised in rabbit (SIGMA C 7930, diluted 1:800), 0.2% triton X100, 0.1% sodium azide (SIGMA) and 1% NGS. After overnight incubation at 4 °C, the cells were washed 3 times in PBS and incubated with goat anti rabbit IgG conjugated to FITC (1:800, FI-1000, Vector, Burlingham, USA) for 90 min. The cells were mounted onto silanized slides and covered with vectashield H-100 (Axxora, Loerach, Germany) sealed with a cover slip and nail varnish.

The specificity of the binding of the GH antibody was determined by a 3 h pre-incubation of the antibody solution with 10 μg/ml bovine GH (H070/H, Batch B980173, BioGenesis, New Fields, UK,) and 0.5% BSA before the application on the cells. Furthermore, in all immunocytochemical setups, the primary antibody was omitted in one sample of PHA-M-stimulated lymphocytes to estimate the nonspecific binding of the secondary antibody.

The GH specific fluorescent signal was completely abolished, when the primary antibody was omitted (Fig. 1A). Moreover, preincubation of the GH-antibody with bovine GH for 3 h abolished the