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Proteomics screening of molecular targets of granulocyte colony stimulating factor in the mouse brain and PC12 cell line

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ABSTRACT

Aims: Granulocyte colony stimulating factor (G-CSF), a new neuroprotective agent, binds to its specific receptors in the brain. In this study we hypothesized that at least a part of G-CSF's neuroprotective effect may be mediated through its interaction with other proteins in the brain.
Main methods: Using an immunoprecipitation (IP) kit, at first the antibody of G-CSF was covalently crosslinked to protein A/G agarose. Then the mouse brain or PC12 cell lysate mixed with G-CSF was added to the agarose beads plus antibody. After immunoaffinity isolation of target proteins, gel electrophoresis was performed and protein bands were identified using MALDI-TOF/TOF and MASCOT software.
Key findings: Our data show that G-CSF physically binds to cellular proteins like sodium/potassium-transporting ATPase, beta actin, aldehyde dehydrogenase, regucalcin and glutathione-s-transferase. These proteins are involved in membrane transportation, cell structure, signal transduction, enzymes involve in calcium related cell signaling and redox homeostasis.
Significance: Interaction of G-CSF with these proteins can explain some of its pharmacological effects in the CNS.

Introduction

G-CSF is one of the hematopoietic growth factors in the cytokine family, which mainly affects the neutrophilic granulocyte lineage. G-CSF is currently prescribed for neutropenic patients. Neuroprotective effects of G-CSF have already been shown in different animal models of cerebral ischemia (Komine-Kobayashi et al., 2006). In several investigations it has been reported that the administration of G-CSF significantly reduced mortality rate and infarction volume, and also improved neurological behavior (Schäbitz et al., 2003).

It is shown that different cells like stromal cells in bone marrow, fibroblasts, macrophages, astrocytes and endothelial cells are able to produce G-CSF in response to a range of stimuli (Malipiero et al., 1990; Demetri and Griffin, 1991; Aloisi et al., 1992; Gimsa et al., 2013). Pharmacological effects of G-CSF are mediated through binding to G-CSF receptor (G-CSFR) which is present on hematopoietic cells or, as recently shown, on neuronal and glial cells (Shimoda et al., 1993; Schäbitz et al., 2003).

G-CSF and its receptor are expressed in the CNS and cerebral microvessels, suggesting G-CSF's autocrine neuroprotection in response to brain damage. Moreover it is established that G-CSF is able to pass through intact blood brain barrier via receptor-mediated endocytosis (Schneider et al., 2005).

Like other growth factors, G-CSF is an anti-apoptotic protein. It is also shown that G-CSF exerts some other beneficial effects like inhibition of inflammation, induction of neurogenesis, migration of hematopoietic stem cells to the injured region, increasing endothelial proliferation and vascular surface, angiogenesis and excitoprotection, which are all involved in G-CSF's neuroprotective effects in the brain (England et al., 2009). Also it seems that G-CSF can be effective in the treatment of neurodegenerative diseases like Parkinson and Alzheimer (Huang et al., 2007; Tsai et al., 2007). In this study we hypothesized that at least a part of G-CSF pharmacological activities depends on its physical interaction with cellular proteins.

Material and methods

Reagents

Recombinant human G-CSF was obtained from Pooyesh Darou, Iran. Anti-G-CSF antibody was purchased from Santa Cruz Biotechnology

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(CA, USA). Pierce Crosslink Immunoprecipitation Kit (Cat. N: 26147), contains sufficient reagents to perform 50 reactions using 10 µl of immobilized antibody support: Pierce Protein A/G Plus Agarose, 20× Coupling Buffer, DSS (disuccinimidyl suberate), IP Lysis/Wash Buffer, 20× Tris-Buffered Saline, and Lane Marker Sample Buffer.

Pierce Control Agarose Resin was purchased from Thermo Scientific Company.

Animals

Twelve BALB/c mice weighing 20–25 g were killed by decapitation. Brains of mice were removed and washed using 0.9% normal saline solution. Tissues were immediately frozen in liquid nitrogen and transferred to −80 °C until use.

Cell culture

After a brief centrifugation and medium removal, ice cold lysis buffer (from immunoprecipitation kit) was added to cells and incubated on ice for 5 min. Lysates were centrifuged at 13,000 ×*g* for 10 min to pellet the cell debris. Total protein concentrations were measured using BCA protein assay kit and bovine serum albumin as standard.

Preparation of tissue extracts

200 mg of the brain tissues was homogenized in 1 ml extraction buffer containing 50 mM Tris, pH 7.4, 2 mM EGTA, 2 mM EDTA, 2 mM Na_3VO_4, 1% Triton X-100, and 10 mM 2-mercaptoethanol and with the further addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) using a Polytron Homogenizer (Kinematica, Switzerland) for 10 s and sonication for 40 s (UP100H, Hielscher). Homogenates were centrifuged (Hettich Universal 320R, Germany) at 25,000 ×*g* for 10 min at 4 °C. Supernatants were removed and stored on ice. Total protein concentrations were measured using BCA protein assay kit (BioRad).

Immunoprecipitation protocol

Pierce Crosslink IP Kit was used to immunoprecipitate G-CSF targets. Briefly, about 20 µg G-CSF antibody was covalently crosslinked to protein A/G linked agarose beads using disuccinimidyl suberate (DSS). Tissue or cell lysates were added to control resin, to minimize unspecific interactions of G-CSF with stationary phase without antibody. G-CSF was added to antibody-crosslinked resin (immunoprecipitation step). Total protein concentration was adjusted to 500 µg in 500 µl.

The target proteins in the cell lysates were enriched by immunoprecipitation using anti-G-CSF antibody. After few washes, proteins were eluted using elution buffer (pH 2.8).

All process was performed according to the data sheet instruction of Pierce Crosslink Immunoprecipitation Kit.

Gel electrophoresis

Freeze dried samples were dissolved in 2×-SDS solution. 20 µl of samples was loaded on a 12% SDS-PAGE and electrophoresis was performed.

In gel digestion

Gel slices were incubated in destaining buffer (50 MeOH, 5% acetic acid) overnight at room temperature. Destaining was continued for 2 more hours by adding fresh buffer for 2 more hours. Gel slices were dehydrated in acetonitrile for 30 min and dried in vacufuge. Gels were covered with reducing buffer (1.5 mg/ml in 100 mM ammonium bicarbonate) for 1 h. Protein alkylation was performed by incubation of gel slices in 100 µl of 10 mg/ml iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature. Gel slices were washed using 0.5 ml of 100 mM ammonium bicarbonate. Gel slices were dehydrated using acetonitrile and dried in vacufuge. 50 µl of 20 µg/ml trypsin was added to each gel slice and incubated overnight at 4 °C. Peptides were extracted in 3 steps by adding 100 µl of 100 mM ammonium bicarbonate, 100 µl extraction solution (50% acetonitrile and 5% formic acid) and finally 150 µl extraction solution. Samples were dried down to final volume of 15 µl in vacufuge. Samples were desalted using ZipTip® µC-18 (millipore). Eluted samples were stored at −20 °C until use.

Mass analysis

Mass analysis was carried out in Genome Research Centre at the University of Hong Kong using 4800 MALDI-TOF/TOF analyzer (ABI). In house MASCOT searching engine was used to analyze mass data. Data were blasted against both NCBInr and SwissProt databases. MASCOT parameters were set as follow: taxonomy: mouse/or human, fixed modification: carbamidomethyl (C), variable modification: oxidation (M), MS/MS fragment tolerance: 0.2 Da, precursor tolerance: 75 ppm, peptide charge: +1, and monoisotopic. MASCOT cutoff scores were set to 30. Furthermore, only top score peptides with P-value smaller than 0.05 were accepted.

Results

In this study immune-precipitation was performed to purify G-CSF interacting proteins. To re-use columns several times, G-CSF antibody was crosslinked to stationary phase. Unspecific interactions were minimized by incubating tissue or cell lysates with control agarose beads alone. Then unbound proteins were used for the rest of the protocol.

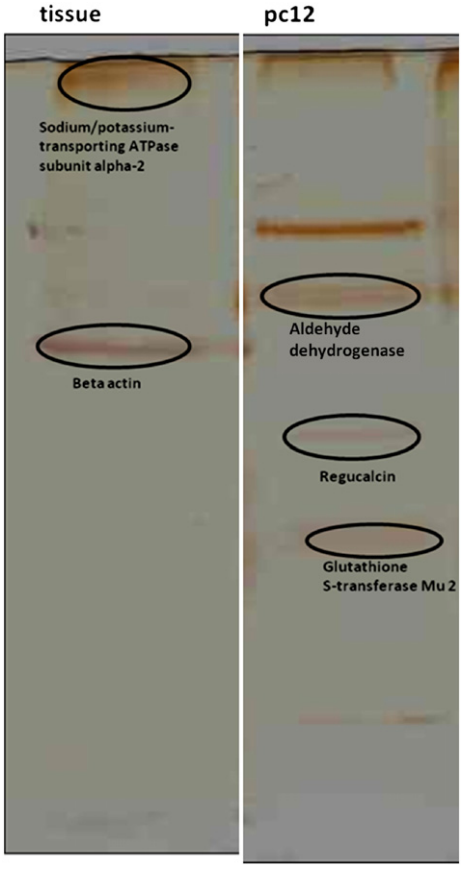

Fig. 1. Target proteins of G-CSF in brain tissue and PC12 cells. This figure shows that G-CSF can efficiently bind to proteins like sodium/potassium-transporting ATPase subunit alpha-2 and beta actin in mouse brain tissue and aldehyde dehydrogenase, regucalcin and glutathione-s-transferase Mu2 in PC12.

Table 1
G-CSF targets in mouse brain and PC12 cell line.

	Protein name	Accession no.	Protein score	Protein score C.I. %	Predicted protein MW	Protein MW	Protein PI	Protein sequence coverage
1	Sodium/potassium-transporting ATPase subunit alpha-2	sp|P06686|	115	100	112,897	113,457	5.39	1%
2	β actin	sp|O18840|	605	100	42,000	42,051.9	5.29	16%
3	Aldehyde dehydrogenase, mitochondrial	sp|P81178|	216	100	34,000–44,000	54,812.6	9.06	4%
4	Regucalcin	sp|Q03336|	372	100	33,406	33,938.6	5.27	10%
5	Glutathione s-transferase Mu2	sp|P08010|	271	100	25,745	25,857	6.9	4%

Fig. 1 and Table 1 show that G-CSF binds to sodium/potassium-transporting ATPase subunit alpha-2 and beta actin in the mouse brain tissue and aldehyde dehydrogenase, regucalcin and glutathione-s-transferase Mu2 in PC12 cells.

Discussion

This study was designed to find possible cellular targets of G-CSF in the CNS besides its receptor. Briefly G-CSF was added to PC12 and brain homogenates. This direct adding of G-CSF to homogenates was performed instead of primarily treating mouse or cell line with G-CSF because we wanted to have considerable amount of G-CSF around neural cells. Because of the high concentration of G-CSF it was possible to detect protein targets more clearly. G-CSF and its target proteins were immunoprecipitated using crosslinked antibody protein A/G-agarose complex. Target proteins were eluted and run and loaded on SDS-PAGE (Cuatrecasas et al., 1968). Protein bands were identified using MALDI-TOF/TOF and MASCOT software. Our data revealed that G-CSF interacts with different proteins participating in cell structure, membrane transporters, enzymes involve in ATP and redox homeostasis and signal transduction.

Occasionally besides the medically predicted action of drugs, there are some other biological effects. These effects cannot be explained with regard to the known mechanism of action of the drug. Proteins that physically bind to other proteins, drugs and small ligands can be isolated in a process called affinity based target deconvolution (Firouzi et al., 2014).

G-CSF is a known hematopoietic growth factor which is used in the treatment of neutropenia followed by hematologic disease, or cancer chemotherapy. G-CSF stimulates proliferation, survival and differentiation of the neutrophilic granulocyte lineage. G-CSF exerts its beneficial effects via binding to its specific surface receptor which is present on different cells like hematopoietic, neuronal and glial cells (Demetri and Griffin, 1991). G-CSF interaction with its receptor leads to the activation of different intracellular signaling pathways that can explain its pharmacologic action in granulocytes. It is reported that G-CSF also exerts a great neuroprotective effect in a variety of animal models of brain injury. However the mechanisms through which G-CSF could act as a neuroprotective agent are not completely known (Solaroglu et al., 2006). Different investigations revealed that G-CSF has anti-inflammatory, anti-apoptotic and excitoprotective effects and it is able to stimulate the mobilization of the stem cells to the injured region of the brain and promotes neurogenesis and angiogenesis (Konishi et al., 1993; Schäbitz et al., 2003; Gibson et al., 2005; Schneider et al., 2005; Komine-Kobayashi et al., 2006; Solaroglu et al., 2006). In this study we found that G-CSF directly interacts with sodium/potassium-transporting ATPase subunit alpha-2 and beta actin in the mouse brain tissue and aldehyde dehydrogenase, regucalcin and glutathione-s-transferase in PC12 cells.

One of the proteins identified as G-CSF target in the brain was β-actin. Six isoforms of actin including β-actin are present and expressed in mammalian cells (Rubenstein, 1990). It is thought that one-half to two-thirds of the cytoplasmic actin in the mammalian brain are β-actin. It contributes to the normal brain structure and function and has role in motor neuron function and axonal regeneration (Choo and Bray, 1978; Otey et al., 1987; Cheever et al., 2011). In a study performed in 2011 they showed that CNS-specific β-actin knock-out mouse exhibited prenatal lethality to some extent. Meanwhile survivors exerted restricted histological abnormalities especially in the hippocampus and cerebellum. Moreover some defects localized in axonal crossing of the corpus callosum have been reported. These findings correlate with the observed hyperactivity, cognitive and maternal behavior impairments (Cheever et al., 2012). Many neuroprotective studies showed that G-CSF can improve cognitive and memory impairments in animal brain injury models (Tsai et al., 2007; Sanchez-Ramos et al., 2009). We may postulate, at least in part, this beneficial effect of G-CSF is mediated via its modulatory interaction (probably enhancing effect) with β-actin.

Sodium/potassium-transporting ATPase protein uses energy from ATP (adenosine triphosphate) in order to transport charged ions into and out of the cells. More specifically, Na^+/K^+ pumps sodium ions (Na^+) out of the cells and potassium ions (K^+) into the cells. These pumps help the transport and maintenance of resting potential, and regulate cellular volume. Na^+/K^+ ATPases also act as signal transducers/integrators to regulate MAPK pathway, ROS and intracellular calcium (Doi and Iwasaki, 2008). Na^+/K^+-ATPases consume almost 2/3 of nerve's energy as compared with 1/3 in other cells (Swann, 1982; Arnaiz, 2007). These transporters are necessary for normal function of the nerve cells and also have an important role in neurotransmission regulation. It also has been shown that there is a correlation between brain edema and Na^+/K^+-ATPase population in the head injury. Activation of MAPK pathway, which is important for neuron survival, in CNS after exposure to G-CSF has already been reported (Abe and Saito, 2000). Our data showed that G-CSF binds to sodium/potassium-transporting ATPase subunit alpha-2. The ability of G-CSF to prevent neural death, reduce brain edema and activation of MAPK signaling pathway can be explained by sodium/potassium-transporting ATPase interfering.

G-CSF physically interacts with aldehyde dehydrogenase (ALDH). Aldehyde dehydrogenases superfamily enzymes have an important role in the metabolism of aldehydes of both endogenous and exogenous origins (Koppaka et al., 2012). Enzymatic activity of ALDHs is important for cellular homeostasis by removing reactive aldehydes derivative from lipid peroxidation (Koppaka et al., 2012). It is shown that a number of aldehydes occur in brain tissue (Amir, 1978). For example succinic semialdehyde is produced from γ-aminobutyric acid metabolism in the brain (Amir, 1978). Amplified or suppressed ALDH activity has been shown in a variety of disorders. So inhibitors or activators of ALDH can represent an approach for the treatment of some pathological conditions in the brain. It is implicated that ALDHs can influence neural function, mainly in dopaminergic nerves. Some isozymes of this super family interact with dopamine metabolism. Accumulation of neurotoxic dopamine metabolites, like 3,4-dihydroxyphenylacetaldehyde, will occur by inhibiting these isozymes. It can contribute to the pathogenesis of Parkinson's disease. Neuroprotective effect of G-CSF has been established in the induced dopaminergic neuronal death (Huang et al., 2007). Recent studies demonstrated that ALDH activation or inhibition can strongly be related to stroke-associated cerebral ischemia injury (Sun and Ren, 2013).

The protective effect of G-CSF on dopaminergic neurons in Parkinson disease models and its potential use in stroke treatment can be explained, in part, via its interaction (including activation or inhibition) with ALDH as a possible molecular target in the brain.

Regucalcin, a calcium binding protein, is another target of G-CSF in PC12 cell line. This protein has regulatory role in the intracellular signaling system and Ca^{2+} homeostasis. Regucalcin controls the increased cell proliferation due to hormonal stimuli (Hamano and Yamaguchi, 1999). Regucalcin is expressed in the CNS neurons, and regulates the accumulation of calcium in the neuron microsomes. Regucalcin has inhibitory effect on protein kinases and protein phosphatases that their activities depend on Ca^{2+} signaling (Hamano and Yamaguchi, 1999; Tobisawa and Yamaguchi, 2003). Also regucalcin inhibits NO synthase activity. Thus G-CSF is able to modulate neuronal homeostasis and function (Tobisawa and Yamaguchi, 2003). G-CSF has neuroprotective effect on excitotoxicity, neuronal death due to increased intracellular Ca^{2+}, in the CNS (Schäbitz et al., 2003; Han et al., 2008). It is possible that G-CSF has effects on intracellular Ca^{2+} concentration by inducing regucalcin activity.

Glutathione-s-transferases (GSTs) are a large family of cytosolic isoenzymes. These multifunctional proteins are essential for detoxication of exogenous toxic that increases the levels of free radicals, like reactive oxygen species. These enzymes conjugate electrophilic compounds to GSH for inactivation and excretion. In the brain, GSTs are located in the glial and neuronal cells and may protect neurons with low GSH against oxidative insults (Salinas and Wong, 1999; Yang et al., 2002; Björk et al., 2006). We showed that G-CSF interacts with GST. Some investigations show that G-CSF is able to affect SOD and GSH-Px activities and consequently inhibits lipid peroxidation in neural tissue injuries (Sanli et al., 2010).

Conclusion

Our study shows that there are some cellular proteins which could physically interact with G-CSF in the brain or PC12 cell line. Identified target proteins were β-actin, sodium/potassium-transporting ATPase subunit alpha-2, aldehyde dehydrogenase, regucalcin and glutathione-s-transferase Mu2. These protein molecules may explain some of G-CSF's properties in the brain. Further activity studies are needed to reveal the probable inhibitory or agonistic effect of G-CSF on these protein targets.

Conflict of interest

There is no conflict of interest.

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