Cloning of Bovine Macrophage Colony-stimulating Factor

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ABSTRACT : Macrophage colony-stimulating factor (M-CSF) is a growth factor required for growth and differentiation of mononuclear phagocyte lineage. Total and 16 poly (A) mRNA of bovine M-CSF were isolated from healthy bovine peripheral mononuclear cells stimulated by phobol 12-myristste 13-acetate (TPA). The more compatible cultured mononuclear cells were 5×10/ml for RNA isolation. TPA-activated mononuclear cells increased the level of M-CSF-mRNA more than concanavalin A (Con A) and lipopolysaccharide (LPS). The optimal analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) for14 Macrophage colony-stimulating factor (M-CSF) as a growth factor required for bovine M-CSF was denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute for 30 cycles. The size of cDNA of bovine M-CSF by RT-PCR was 774 base pairs. A 774 base pairs cDNA encoding bovine M-CSF was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR). Ligated cDNA was transformed to competent cells and then plasmid isolation and digestion was performed. Molecular cloning and sequencing were performed for cDNA of bovine M-CSF. The size of cloned cDNA of bovine M-CSF was 774base pairs. The homology of base sequence and amino acid sequence was 88% and 86% compared with known human M-CSF, respectively. From a high degree of sequence similarity, the obtained cDNA of bovine M-CSF is thought be a specific gene of bovine M-CSF. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 6 : 892-897)***

Key Words : M-CSF, RT-PCR, Cattle

INTRODUCTION

Macrophage colony stimulating factor (M-CSF) has long been recognized as the most strong immunomodulator (Motoyoshi et al., 1983; Stanley et al., 1983). M-CSF directly affects monocytes and macrophages to produce the granulocyte-CSF (G-CSF), granulocyte/ macrophage-colony stimulating factor (GM-CSF) and also affects monocyte to enhance the tumor killing activity. Given these information, it was recognized as useful edicine source (Das and Stanley, 1982; Motoyoshi et al., 1983; Morgan and Stanley, 1984; Met-calf, 1986; Motoyoshi et al., 1986; Horiguchi et al., 1987; Horiguchi et al., 1988; Antczak and Gorman, 1989; Andreani et al., 1991).

Human M-CSF is a lipoprotein with 85 kDa of molecular weight and is known as a hemopoietic factor to activate the monocyte, neutrophil and thrombocyte. It exhibits several other effects such as reduction of blood cholesterol level, placental formation, maintenance of pregnancy, amplification of monocyte antitumor activity etc (Bartocci, 1983). Studies related to cloning, expression and clinical applications of human and mouse M-CSFs were reported (Stanley and Heard, 1977; Guilbert and Stanley, 1980; Hanamura et al., 1980; Ralph et al., 1980; Das et al., 1981; Tushinski et al., 1982; Chen et al., 1983; Stanley et al., 1983; Bartelmez and Stanley, 1985; Rettenmer et al., 1986; Horiguchi et al., 1987; Rambaldi et al., 1987; Rambaldi et al., 1988; Sariban et al., 1988) but research related to the separation, purification, cloning, expression and application of M-CSF of cattle was very few (Oshima et al., 2003; Yoshihara et al., 2003).

Application of bovine M-CSF would be rather broader than that of human M-CSF (hM-CSF). These might be included the analysis of etiology, prevention, and indicator of treatment for mastitis, infectious diseases and metabolic diseases, giving a serious damage to cattle. Hence, in the present study, we cloned bovine M-CSF.

MATERIALS AND METHODS

Isolation and purification of total RNA and mRNA from Cattle M-CSF

Peripheral blood (200 ml) of healthy cattle (Holstein, ♀) was collected and centrifuged (4°C) to isolate total RNA. Mononuclear cells were separated and collected from the buffy coat layer harvested after centrifugation.

These cells were used to determine the appropriate levels of macrophage stimulants and the cell numbers. Concanavalin A (Con A, Sigma-Aldrich Co.), phorbol 12-myristate 13-acetate (TPA, Sigma-Aldrich Co.) and lipopolysaccharide (LPS, Sigma-Aldrich Co.) were used as the stimulants.

Various levels of the mononuclear cells were grown in the medium of 10% FCS RPMI 1640 (w/v) 10% FCS at a

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5% CO_2 incubator for 24 h. Only adherent cells were collected, cesium trifluoroacetate (Sigma-Aldrich Co.) treated, and finally purified by ethanol precipitation. The concentration was determined by spectrophotometric method (Manchester, 1995; Wilfinger et al., 1997). mRNA was purified from total RNA using a oligo (dT) cellulose column and then spin column chromatography (Union $_{32}$R plus, Hanil, Kor.).

Conditions for 5' and 3' primers synthesis

Primers were synthesized from four amino acid sequences (301, 302, 303, 304) of human M-CSF which are identified amino acid of M-CSF sequence. Simulatneously, forward primer 5' GAA CAG TTG AAA GAT CCA and GTG, and reverse primer 5' TCG GAC GCA GGC CTT GTC ATG 3' (Perkin-Elmer Co. USA) were used.

Already purified Total RNA and mRNA were used as templates.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR condition was determined for each primer and product from each synthesized primer (base combinations) as well. Simultaneously, RT-PCR conditions for denaturation, annealing, extension and cycling were also carried out. PCR product being produced was vortexed, spin-downed to remove the mineral oil, added chloroform(99%, 100 μl) and vortexed for 5 min. DNA was collected from this mixture by the low temperature centrifugation (100 g) and stored suspended DNA (60 μl) at -20°C.

Detection of PCR products

PCR products were separated by agarose gel (2%) electorphoresis using 0.5% TBE buffer consisting of tris (hydroxymethyl) aminomethane (>99%), boric acid (>99.5%), and Na_2 EDTA·$2H_2O$. The products being stained with ethidium bromide (EtBr) were detected under UV. The λ/Hind III was used as a marker.

Southern hybridization

The PCR products (cDNA) marked in the under UV was denatured in a 0.4 N NaOH solution for 15 min. Finally, the marker was southern-transferred onto the Hybond N+membrane (Amersham Co. USA) for 12-16 h. The membrane transferred was neutralized in a 2×SSC solution for one min, and dried with 3 mm filter paper, and then stored, wrapped with a 3 mm filter pater until use. Before hybridization of the product, it was prehybridized in the solution of prehybridizing buffer consisting final concentration of 5×SSC solution, 5×Denhardt's solution, and 0.5% (w/v) SDS solution.

To the sealing bag containing the transferred membrane was added hybridization buffer and electrically sealed. More than 2 h after incubation of this bag in a water bath (37°C), labelled M-CSF probe DNA was added and continuously shaked in a shaking water bath (37°C) for overnight, After hybridization, the bag was cut and substantial amount of hybridization solution was aspirated. Hybridized membrane was mixed in a solution of 2×SSC/0.1% SDS (250 ml) at 37°C for 10 min to remove excess 32p. This process was repeated until no 32p was detected by Aloka (TGS-121) survey meter (monitor). The hybridized membrane was autoradiographied.

Electrophoresis for cDNA gel extraction

Electrophoresis was performed as mentioned above using 2% agarose gel and 1×Tris-acetate (TAE) buffer except for using a bromophenol blue (BPB) as a dye, 90 V and 150 mA. cDNA band was immediately confirmed and cut under UV after electrophoresis, and put a sterile eppendorf tube (1.5 ml) for extraction of the DNA.

Gel extraction for cDNA fragments

Extraction of the cDNA contained in the gel was performed according to manufacture's instruction (Qiagen Co. Kit, USA). The cDNA was precipitated in ethanol, concentrated and stored (5 μl) at -20°C until use for cloning.

Cloning of M-CSF cDNA

Cloning of M-CSF cDNA was conducted by Cloning kit (Invitrogen Co., USA).

Before cloning, considered the insert DNA and its size, cloning vector of pGEM (3 Kb) was diluted with TE buffer. The cDNA cleavaged with EcoRI was ligated into the plasmid at 12°C for overnight in the reaction mixture consisting double distilled water (4.5 μl), 10×ligation buffer 1 (1 μl), diluted vector (2 μl), insert DNA (2.5 μl), T4 DNA ligase (1 μl). Transformation was performed by transfection of the ligated reactants to competent cells in the presence of 2-mercaptoethanol (2-ME).

Transformants were grown on the LB agar plate which consisted of bactotryptone, bacto-yeast extract, NaCl and 5-bromo-4-chloro-3-indolyl-β-D-galactoside(IPTG), and only white colonies transplated onto the new LB agar plates or into sterile Asist tube and incubated in a shaking incubator (37°C). Pure white colonies were collected and plasmids were separated. The white colonies was cultured in 2 ml LB medium containing Kanamycin (50 μg/ml) with shaking.

Separation of plasmids

For Separation of plasmids, 1-1.5 ml of the medium containing transformants was used.

The plasmids separated were identified with 2% agarose gel electrophoresis.

Figure 1. The cDNA blot of bovine M-CSF by immobilization analysis.

Plasmid digestion

Plasmid (insert cDNA) digestion was performed in a 15 μl volume containing the isolated plasmid (5 μl), 10×digestion buffer (1.5 μl), EcoR1 enzyme (10 μg/μl) (0.5 μl), and distilled water (8 μl).

cDNA sequencing

The cDNA was incubated in a 2 N NaOH/2 mM EDTA solution for 30 min in a water bath (37°C), and denatured by 3 M NaOAc (pH 4.4) solution and absolute ethanol. After these processes, annealing labeling reaction and termination were basically followed the instruction of sequenase Kit (version 2.0, labeled dCTP, USA). After termination, Polyacrylamide gel (7%) electrophoresis was performed for 90 min with loading volume (2.5 μl) and 1,500 volt and then sequenced by autoradiography.

RESULTS

Optimum number of mononuclear cells

The Optimum number for this experiment was found to be 5×10^6/ml.

Effects of stimulants

The expression of M-CSF mRNA treated by TPA (40 ng/ml) was larger than the treatment of Con A (10 μg/ml) and LPS (10 μg/ml).

Condition for Reverse transcriptase-polymerase chain reaction (RT-PCR)

Optimum condition was found to be 30 cycles with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min.

cDNA of PCR products

A PCR product of 774 bp was obtained from 30 cycles operated by denaturation at 94°C for one min, annealing at 57°C for one min and extension at 72°C for 1 min (Figure 1).

cDNA gel extraction

cDNA contained in the specific band obtained by RT-PCR was extracted from the gel and was exactly same as reported (Yoshihara et al., 1998).

cDNA cloning of M-CSF

The cDNA identified by 2% agarose gel electrophoresis was found to be 774 bp.

cDNA gene sequencing

Sequence of the cDNA revealed that the cDNA size was appeared to be 774 bp (Figure 2). Its homology was 88% and 86% in DNA base sequence and amino acid sequence with human M-CSF (Rambaldi et al., 1987; Rambaldi et al., 1988).

DISCUSSION

Cytokines are cellular soluble factors to induce proliferate and differentiate of cells. Variety cytokines including the M-CSF entered into cells via receptors expressed on the cell surface of target cells, and then exerts several biological activities. Several powerful functions of M-CSF were known, so that its application to cattle has important meanings. It is very hot topic to study isolation, purification and expression of human M-CSF (hM-CSF) since it is a hemopoietic factor and involved in the lipid metabolism and embryo development as well. Moreover monocytes and macrophages exhibits antitumor activities so that it is positivie for M-CSF to provides antitumor mechanism in the body; thus currently, its isolation, purification and expression are being extensively studied (Ralph et al., 1980; Motoyoshi et al., 1983; Motoyoshi et al., 1986; Horiguchi et al., 1987; Rambaldi et al., 1987; Horiguchi et al., 1988).

It was proven that when monocyte of human peripheral blood was cultured with hM-CSF, it exhibited very high antitumor activity for human leukaemia cell lines K562, 16 HL60 and U937 (Horiguchi et al., 1987).

Rambaldi et al. (Rambaldi et al., 1988) reported that in the expression experiment of human M-CSF isolated 36 from peripheral blood, better expression results was obtained from incubation for 2 h with 1×10^6 cells/ml of mononuclear cell. This result was omewhat different from our result of 5×10^6/ml, but it is not clear whether this was

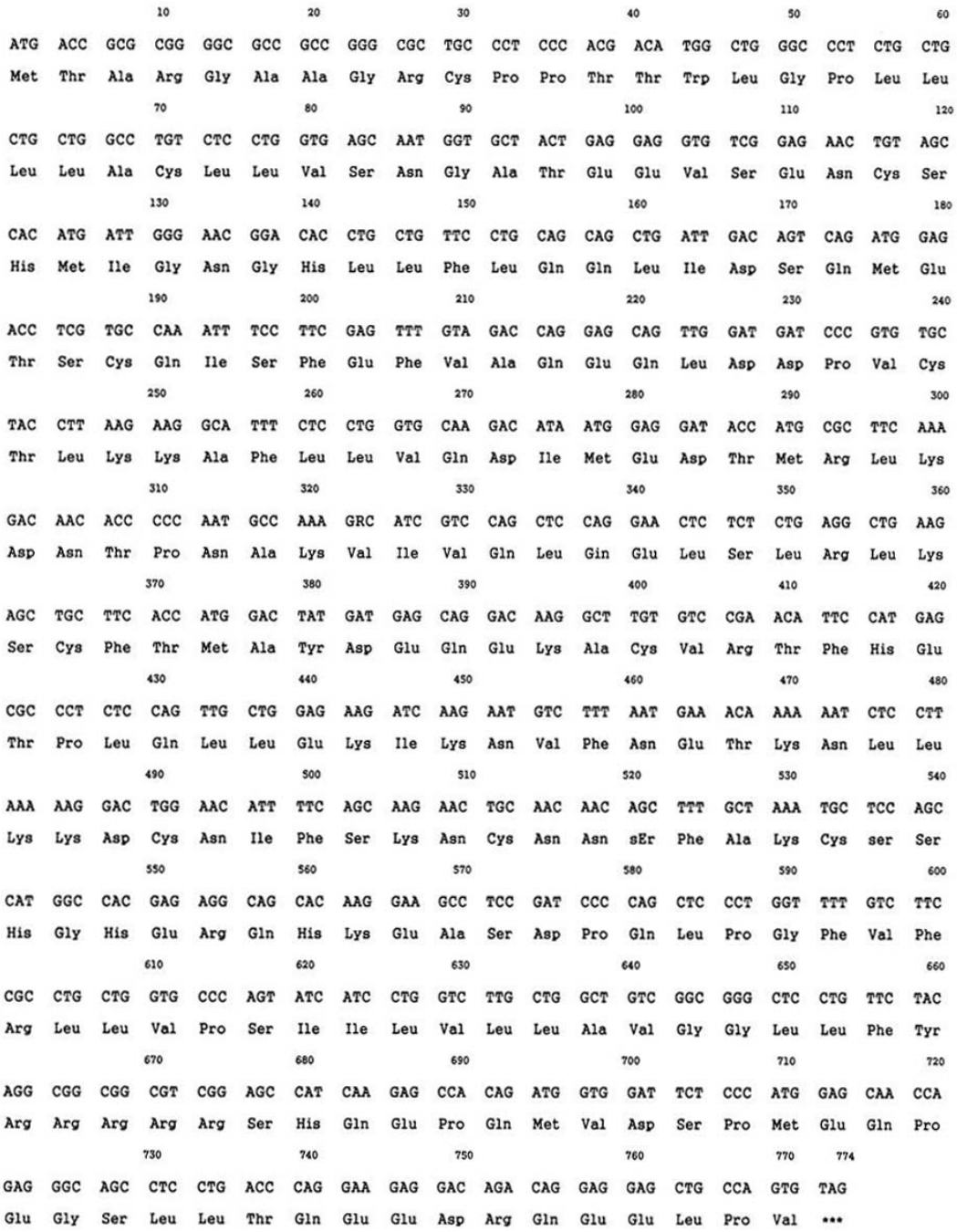

Figure 2. Neucleotide sequence of bovine macrophage colony-stimulating factor cDNA gene.

resulted from stimulation differences between macrophages of human and cattle. It might be a reason that Percoll 40 of isolation solution used in this study was different from Ficoll-Conray used by Rambaldi et al. (1998).

Rambaldi et al. (Rambaldi et al., 1987; Rambaldi et al., 1988) reported that Y-interferon (Y-IFN) 500 U/ml or phorbol myristate acetate (PMA) 10^{-10} Mol/L released from activated T cell and NK cell was suitable for macrophage activation. Horiguchi et al. (1987) also reported that maximum RNA was produced in human peripheral blood M-CSF when stimulated for 6 hrs with TPA. Sariban et al. (Sariban et al., 1988) performed an experiment to stimulate human tumor necrosis factor (TNF) gene expression of monocytes derived from healthy human peripheral bloods with TPA, cycloheximide (CHX), an inhibitor of protein synthesis, and actinomycin D (ACT), an antitumor agent. They found that RNA amount of TNF stimulated by ACT (5 μg/ml) for one h was rather decreased to 25%, and by CHX (10 μg/ml), no RNA detected; however, by TPA (32 nM), increased to 50 fold. These results suggested that TPA is a

suitable stimulant for M-CSF and TNF as well.

The M-CSF gene size cloned from peripheral blood of cattle was 774 bp.

No report was appeared in the literature, so it very difficulties to interpret this result at this moment; however even its size is somewhat different from known human M-CSF gene size 4.0 or 4.6 Kb, they exhibited 88% homology in base sequence and 86% homology in amino acid sequence. This suggests that the 774 bp gene could be a bovine specific M-CSF gene (Ralph et al., 1980; Das et al., 1982; Kawasaki et al., 1985; Horiguchi et al., 1987). We believe that even though the 774 bp obtained from this study is not full gene size of bovine M-CSF gene, it is necessary to investigate the expression and functions of M-CSF gene, due to its several important biological functions. New techniques for genomic DNA separation and aquisition are required other than currently proceeded RT-PCR for cDNA aquisition.

Blood activity fo normal human M-CSF (hM-CSF) was turned out 174±76 U/ml (about 40 bM) assayed by radioimmunoassay (RIA) (Das et al., 1981) and 5.5±1.1 ng/ml assayed by enzyme-linked immunosorbent assay (ELISA) (Hanamura et al., 1988).

Simultaneously, blood activity of pregnant women was not consistent: its value was elevated by pregnancy, increased to 2-3 fold at the late stage of pregnance, decreased by parturition, and finally returned to original value after 2-3 weeks later of parturition (Hanamura et al., 1988). In a mouse experiment, normal mouse treated with gonadotropin has an increased uterus weight as well as M-CSF activity; however in the ovariectomied mouse treated with gonadotropin no such effect was observed, facts that gonadotropin stimulates M-CSF growth in uterus via ovary (Guilbert and Stanley, 1980). Monocytes of human peripheral blood cultured with hM-CSF exhibited an increase antitumor activity against human leukemia cell line (K562, 35 HL60, U937, etc) (Horiguchi et al., 1987).

As mentioned, the hM-CSF acts not only as a hemopoietic growth factor, but is tightly related to lipid metabolism and development of fetal. Furthermore, it induced antitumor activity of monocytes and macrophages for body defence mechanism.

In animals, estrus cycle and spleen macrophage activity of rats during pregnancy, Kang and Kwak (Kang and Kwak, 1994) proved that the macrophage activity was highest (68±6.4%) at proestrus and lowest (49±4.6%) at metestrus, and during pregnancy, the activity was less than 5% at 1 first day, but it was slowly increased from days, rapidly increased around 12 days and continued the increase before delivery. Especially, the reason why macrophage activity was low to 5% at day 1 is might due to ovulation which is thaught to be a big injury.

The activity is being used for cure it, after which the activity is increased due to in response to pregnancy phenomenon (Metcalf, 1986). The activity of spleen macrophage observed from pregnant rats by Kang and Kwok was quite agreed with the fact of increased hM-CSF activity in blood of pregnant during pregnancy investigated by Hanamura et al. (Hanamura et al., 1988) suggesting that M-CSF activity is closely related to macrophage activity. Hence, bovine M-CSF gene expression and its functional tests, macrophage activity could be used to markers for identify causes of mastitis, other infectious disease, metabolic disease and for prognosis of cattle (Newby and Bourne, 1977; Craven, 1986; Colditz and Mass, 1987; Duhamel, 1987; Oshima et al., 2003; Yoshihara et al., 2003).

CONCLUSION

Mononuclear cells were isolated from peripheral blood of cattle for cloning the M-CSF gene of cattle. TPA was best stimulant for mRNA production at mononuclear cell (5×10^6/ml) among other stimulants (Con A, LPS) tested. RT-PCR with template from total RNA and mRNA of the peripheral blood produced a cDNA fragment with 774 bp long, identified by southern hybridization. After gel extraction of cDNA with a band (774 bp), M-CSF cDNA was cloned. Competent cell was trasfected by the vector ligated with cDNA obtained. After incubaion, white colony was collected and digested. Cattle's M-CSF insert DNA (gene) was separated from the digest and sequenced to get 774 bp size of gene. Base sequence of M-CSF gene showed 88% homology with human gene and amino acid sequence was 86% homology with human indicating that this gene might be a specific M-CSF gene of cattle. However, further systemic researches should be performed for the gene expression and the functions of its product.

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