INFLUENCE OF CHEMOTHERAPEUTANTS AND CYTOKINES ON GROWTH AND TRANSGENE EXPRESSION OF BONE MARROW CELLS FROM MT/P210$^{bcr\text{-}abl}$ TRANSGENIC MICE

CHEN Hanchun 陈汉春, Andrew Pierce,[1] Tony Whetton[1]

Research Center for Molecular Biology, Hunan Medical University, Changsha 410078, China
[1]Department of Biomolecular Sciences, UMIST, Manchester, M60, 1QD, UK

ABSTRACT

Objective: To investigate the influence of chemotherapeutic agents and cytokines on growth of bone marrow cells from MT/p210 $^{bcr\text{-}abl}$ transgenic mice. Methods: The bone marrow cells of transgenic chronic myelogenous leukemia (CML) model mice carrying metallothionein (MT) promoter/enhancer, bcr-abl (p210) cDNA and SV40 splicing/poly (A) signal sequences were cultured in liquid and soft agar with hydroxyurea (Hu), 5-fluorouracil (5-Fu), mouse stem cell factor (mSCF) and mouse interleukin-3 (mIL-3) independently or collectively. The cells and colonies were counted. The levels of transgene expression were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Results: The cell proliferation, colony formation and transgene expression of the bone marrow cells were stimulated with mSCF and mIL-3, but there was little growth without any growth factors, or when mSCF, mIL-3 and Hu or 5-Fu were added. Conclusion: The combined utilization of chemotherapeutants and cytokines is a potentially effective strategy of clinical treatment for CML.

Key words: Gene, ABL, Transgenic mouse, Chemotherapeutant, mSCF, mIL-3

An abnormal shorter chromosome 22 called Philadelphia (Ph) chromosome was generated from the reciprocal translocation between normal chromosomes 9 and 22. As a result of the reciprocal chromosomal rearrangement, a chimeric bcr-abl gene appeared at the fusion-point of the Ph chromosome and its expression product was been shown to be an 210kD protein p210$^{bcr\text{-}abl}$ and elevated tyrosine kinase activity. The abnormal activation of the Ras-mediated signaling pathway by p210$^{bcr\text{-}abl}$ protein may lead to unrestricted myeloproliferation and then the onset of chronic myelogenous leukemia (CML).[1] The main treatment strategies for CML include bone marrow translocation (BMT), interferon (IFN) treatment and chemotherapy using Hydroxyurea (Hu) and 5-Fu. BMT is mainly suitable for those patients who are younger than 45 years old and needs a human leukocyte antigen (HLA)-matched marrow donor or purification of the autografts *in vitro*. The simple high-dose chemotherapy usually produces certain side effects such as inhibition of marrow development.[2] Now the combinative therapies with chemotherapeutants and cytokines are being tried to treat CML cases.[3] In this study, the bone marrow cells from MT/p210$^{bcr\text{-}abl}$ transgenic mice were incubated with Hu or 5-Fu and mSCF or mIL-3 independently or together in either a liquid culture or soft agar medium to observe the influence of these chemotherapeutants and cytokines on growth and transgene expression of the CML mice bone marrow cells, and explore the effective approaches of combination-therapy for CML.

MATERIALS AND METHODS

Materials

MT/p210$^{bcr\text{-}abl}$ transgenic C57BL/6XDBA2 mice were bred by Honda, et al.[4] Iscoves medium, fetal calf serum (FCS), mIL-3, Trizol reagent, dNTP and Taq DNA polymerase were products of GIBCO/BRL. Bovine serum albumin (BSA), 5-Fu, Hu, agar, RNase inhibitor DEPC and RNase-free RNaseI were provided by Sigma. Mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) and mSCF were purchased from Genzyme. Titan™ one tube RT-PCR system was obtained from Boehringer Mannheim. PCR primers were synthesized by Pharmacia Biotech and the nucleotide sequences of the primers are as follows:

Accepted for publication: September 22, 1999
This work was supported by the National Natural Science Foundation of China (No.39570805)
Correspondence to: Chen Hanchun, Research Center for Molecular Biology, Hunan Medical University, No.88, Xiangya Road, Changsha 410078, China; Phone: (0086-731)-4474411 ext.2547; Fax: (0086-731)-4471339; E-mail: mbrc@public.cs.hn.cn

bcr sense primer:5'-ATTCGCTGACCATCAATAAG-3'
abl antisense primer:5'-GGCGTGATGTAGTTGCTTGG-3'
SV40 sense primer:5'-CGCTTTGGTCCCGGATCTTT-3'
SV40 antisense primer:5'-TCATCACTAGATGGCATTTC-3'

Methods

PCR analysis of bcr-abl fusion gene bearing by MT/p210$^{bcr\text{-}abl}$ transgenic mice

A piece of 200mg tissue from MT/p210$^{bcr\text{-}abl}$ transgenic mice was taken by punching the ear and added to 20 μl of digestion buffer containing 20 mmol/L NaCl, 1 mmol/L EDTA, 1%SDS, 20mg/ml proteinase K and 50mmol/L Tris-HCl at pH 8.0. The sample was incubated in a water bath at 55°C for 30 min and added to 180 μl of distilled water, then boiled for 5 min after mixing. Bcr-abl and SV40 DNA PCR amplifications were performed in the total volume of 50 μl with 1.0 μl of DNA as templates respectively. The standard program of amplification was as follows: denaturation at 95°C for 30 sec, annealing at 54°C for 45 sec and extension at 72°C for 1 min. The extension procedure was continued at 72°C for 5 min after 35 cycles were completed. 10 μl of PCR products was analyzed by 2.5% agarose gel electrophoresis and stained with ethidium bromide.

Bone marrow cells collection and liquid culture

Bone marrow was collected from the two femores of MT/p210$^{bcr\text{-}abl}$ transgenic mice bearing bcr-abl cDNA sequence and washed once with Iscoves medium. 4.4×10^6 bone marrow cells were incubated in Iscoves medium containing 20% FCS and 10% BSA. Several kinds of chemotherapeutants or cytokines were added into different culture groups: group 1 -without any drugs or factors; group 2 -100 ng/ml mSCF and 20 ng/ml mIL-3; group 3 -additional 10 μg/ml 5-Fu being added to the culture used for group 2; group 4-extra 10mmol/L Hu being added to the culture used for group 2. Triplicate cultures for each mouse were incubated at 37°C for seven days in a fully humidified atmosphere of 5% CO_2 in air. The viable cells were by trypan blue dye exclusion test counted every day; a growth curve of the cells in each culture condition was drawn by using the average cell-number of twenty mice.

Bone marrow cells culture in soft agar medium

The bone marrow cells in different initial density (1.0×10^4/ml to 5.0×10^6/ml) were suspended in 1.0 μl of 0.3% agar culture medium containing Iscoves medium supplemented with 20% FCS, 10% BSA and different kinds of chemotherapeutants and cytokines. By adding different factors for the experimental groups, several culture conditions were as follows: group 1–250 ng/ml mGM-CSF and 20 ng/ml mIL-3; group 2–10 μg/ml 5-Fu being added to the culture used for group 1; group 3–10 mmol/L Hu being added to the culture used for group 1; group 4–100 ng/ml mSCF being added to the culture used for group 1; group 5–10 μg/ml 5-Fu being added to the culture used for group 4; group 6–10 mmol/L Hu being added to the culture used for group 4. Triplicate cultures for each mouse in each culture condition were incubated at 37°C for twelve days in a fully humidified atmosphere of 5% CO_2 in air. Colonies (>50 cells) were counted and the colony-forming curve in each culture condition was drawn according to the average colony-number of twenty mice.

Extraction, purification and concentration measurement of total RNA

The cells in liquid medium after being cultured for seven days were collected by centrifugation and then suspended in 1.0 ml of Trizol reagent. Total RNA was isolated according to the recommended protocols (GIBCO/BRL) and dissolved in 90 μl of DEPC-treated water. 10 μl of 10 × high-salt digestion buffer and 1.5U of RNase-free DNase I were added into the RNA sample. The probable residual DNA in the RNA sample was digested at 37°C for 30 min. The pure RNA was extracted by phenol/chloroform method and dissolved in 20 μl of DEPC-water. RNA concentration was determined by spectrophotometry.

RT-PCR analysis

SV40 mRNA RT-PCR amplification was performed from 1 μg of RNA by using Titan™ one tube RT-PCR system according to the recommended protocols. The reverse-transcription reaction proceeded at 49°C for 30 min. The PCR program was designed as follows: denaturation at 94°C for 30 sec, annealing at 49°C for 40 sec and extension at 68°C for 40 sec. The extension was continued at 68°C for 5 min after 35 cycles were completed. Using Φ X174 DNA/Hinc II fragments, PCR amplification fragments of bcr-abl and SV40 DNA from MT/p210$^{bcr\text{-}abl}$ positive transgenic mice as the molecular size markers, 10 μl of RT-PCR products were separated by 2.5% agarose gel electrophoresis and stained with ethidium bromide. The 174bp band from SV40 mRNA RT-PCR fragments was scanned and quantitated by using Eagle Eye II image recognition and analysis system.

RESULTS

Hereditary Frequency of the Transgene Born by MT/p210$^{bcr\text{-}abl}$ Transgenic Mice

Among 442 descendant mice with MT/p210$^{bcr\text{-}abl}$ transgene, 298 mice (67.4%) bearing bcr-abl cDNA sequence were found. 118 male mice (50%) and 180 female mice (87.4%) bearing bcr-abl cDNA sequence were found in the 236 male and 206 female descendants with the MT/p210$^{bcr\text{-}abl}$ transgene respectively. The hereditary frequency of transgene in female mice was 1.3 times of that in male mice.

Influence of Chemotherapeutic Agents and Cytokines on Growth of Bone Marrow Cells from MT/p210$^{bcr\text{-}abl}$ Transgenic Mice

The bone marrow cells did not proliferate and died rapidly in the culture without any growth factors. Combining SCF with IL-3 can stimulate bone marrow cells proliferation especially on the fourth and fifth day of cultivation. Separate addition of two chemotherapeutic agents mentioned below on the basis of promoting action upon cell proliferation by SCF and IL-3, the cell killing and cell growth suppressing effects of 5-Fu occurred earlier but lasted a shorter time than that of Hu (Figure 1). No colony could be seen in the cultures without addition of growth factors or in those supplement with 5-Fu or Hu. Coordinating with the combinative action of GM-CSF and IL-3, SCF markedly promoted the colony formation (Figure 2).

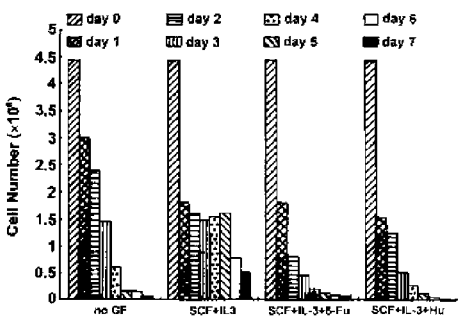

Fig 1. Influence of chemotherapeutic agents and cytokines on the growth of bone marrow cells from MT/p210$^{bcr\text{-}abl}$ transgenic mice

Influence of Chemotherapeutic Agents and Cytokines on Transgene Expression of Bone Marrow Cells from MT/p210$^{bcr\text{-}abl}$ Transgenic Mice

The results are shown in Figure 3. PCR amplification signals of both bcr-abl and SV40 DNA were observed in the expected sizes of 372bp and 244bp respectively (lane 1 and 2). Taking the expression level of SV40 in the uncultivated bone marrow cells from MT/p210 (bcr-abl) transgenic mice as control (lane 3, the band with molecular size of 174bp, its relative expression value=1), the relative expression values of the transgene within bone marrow cells in different culture conditions were as follows: 0.354± 0.008 (lane 4, n=20) in the culture without any growth factors; 0.855± 0.007 (lane 5, n=20) in the culture with IL-3 plus SCF; 0.690±0.006 or 0.642±0.005 (lane 6 and 7, n=20) in the culture with IL-3+SCF+5-Fu or IL-3+SCF+Hu respectively. 0.117±0.008 (lane 8, n=20) in the culture with Hu but without any growth factors.

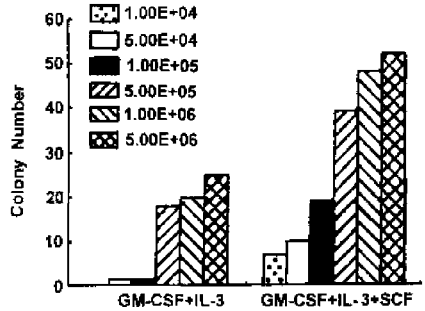

Fig. 2. Influence of cytokines on colony formation of bone marrow cells from MT/p210$^{bcr\text{-}abl}$ transgenic mice

Fig. 3. RT-PCR amplification map of the transgene expressed mRNA in the cultured bone marrow cells from MT/p210$^{bcr\text{-}abl}$ transgenic mice

M: Φ X174/Hinc II DNA molecular size markers

DISCUSSION

With its characteristic Ph chromosome, CML has become the ideal model for studying the molecular pathogenesis and treatment mechanisms of leukemia. It was usually used in the evaluation on treatment effects of various therapeutic agents or strategies.[5] It is also of momentous significance for exploring the new agents or strategies of combinative therapy and studying the molecular mechanisms in clinical treatment of CML. Using transgenic animal models for studying human diseases there are many advantages, such as obtaining materials easily, operating simply and accepting less risk etc. The full bcr-abl cDNA sequence integration could induce CML in the transgenic mice; there was no difference in molecular sizes of PCR products between that from bcr-abl cDNA integrated into genome and that from bcr-abl mRNA of the transgenic mice. In order to detect the expression level of the transgene, a splicing

sequence of 70bp was involved in the integrated SV40 poly(A) signal sequence, so the PCR product of genomic amplification is 244bp whereas that of mRNA amplification is 174bp, which is due to the removal of the 70bp sequence by splicing.[4] This size-difference of 70bp can be utilized to determine whether the RT-PCR products were amplified from DNA or mRNA of the transgene. The analysis of hereditary frequency of the transgene in MT/p210$^{bcr\text{-}abl}$ transgenic mice showed that the female individuals were easier to be involved in CML than the males and suggested that the study on sex incidence of leukemias such as CML deserves to be further explored in the human race.

The current trends of combination-therapy for CML and investigation of its molecular mechanisms include several aspects. Simultaneous with the killing of malignant cells or inhibition of their abnormal proliferation by using chemotherapeutic agents, the normal signal transduction pathways are reactivated *via* multiple-level actions of cytokines; this would promote the normal proliferation and differentiation process of hematopoietic stem cells or progenitors, then the correction of malignant phenotypes in CML would be achieved.[5-8] The results of this study showed that the combinated application of SCF with IL-3 markedly facilitated proliferation of hematopoietic stem cells or progenitors, but this effect was not specific to the regulation of proliferation in normal or malignant hematopoietic cells. The cell proliferation, colony formation and transgene expression of the incubated bone marrow cells from MT/p210$^{bcr\text{-}abl}$ transgenic mice were significantly suppressed by the removal of growth factors or the addition of chemotherapeutant (5-Fu or Hu) in the culture medium. These results further demonstrated that CML cells possessed some special features such as survival superiority, drug resistance and growth factor dependence etc. It is suggested that the combinative application of chemotherapeutants with cytokines can suppress the expression of bcr-abl chrimeric gene, reduce the production of p210$^{bcr\text{-}abl}$ and meanwhile protect the proliferation and differentiation of hematopoietic stem cells or progenitors. The strategy of combination-therapy by using chemotherapeutants and cytokines has opened up brilliant prospects for the improvement of CML treatment. In addition, the study on gene therapy for CML has been also started.[9] Further preclinical studies are necessary to push ahead with the combination-therapy and gene therapy of CML.

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