ANTICANCER RESEARCH *24*: 2739-2744 (2004)

Role of Enzymatically Inactive Procathepsin D in Lung Cancer

VACLAV VETVICKA[1], JANA VETVICKOVA[1] and PETR BENES[2]

[1]*Department of Pathology, School of Medicine, University of Louisville, Louisville, KY 40292, U.S.A.;*
[2]*Faculty of Science, Masaryk University, Brno, Czech Republic*

Abstract. *Procathepsin D is over-secreted by some human cancer cells. This enzymatically inactive precursor has been established as playing an important role in the development of several types of cancer. In the present investigation, we used both the isolated human procathepsin D and a synthetic 44 amino acid peptide corresponding to the activation peptide of procathepsin D to test their effects on the proliferation of lung cancer cells. We showed that both the procathepsin D and the activation peptide act as growth factors. In parallel, we also measured the secretion of procathepsin D by lung cancer cells and compared the secretion with invasiveness through Matrigel membrane. Our findings represent the first experimental data showing the direct effects of procathepsin D and its activation peptide on growth and invasiveness of lung cancer cells.*

Procathepsin D is a major secreted glycoprotein from breast cancer cell lines such as MCF-7 and ZR-75-1 (1), prostate cancer cell line DU145 (2) and ovarian cancer cell line OVCAR-3. Vignon *et al.* first proposed that procathepsin D served as a growth factor for cancer cell lines (3). Moreover, several clinical studies suggested a potential role for this molecule in metastasis, because its concentration in primary tumors correlated with an increased incidence of tumor metastasis (4). In athymic nude mice, it was shown that rat tumor cells were converted from low to high metastatic potential by transfection with the cDNA for human cathepsin D (CD) (5), indicating the role of procathepsin D in metastases.

Based on almost two decades of intensive research showing that procathepsin D is secreted from several types of tumors including breast cancer (6,7), ovarian cancer (8), squamous cell carcinoma of the head and neck (9), endometrial adenocarcinoma (10), colon carcinoma (11), laryngeal tumors (12) and prostate tumors (13), procathepsin D and the fully mature enzyme cathepsin D have been suggested as important factors for the prognosis of several types of cancer. In addition, despite numerous studies suggesting the involvement of CD (14), the direct enzymatic involvement of mature CD in the growth and invasiveness of cancer cells has never been demonstrated (15), therefore strongly supporting the hypothesis concerning the biological significance of procathepsin D (16).

The role of procathepsin D has been demonstrated in at least 12 different types of cancer (16), however lung cancer has not been vigorously studied. Based on the limited knowledge, higher cathepsin D activities were observed in lung tumor cells (17) and can be used for prognosis (18). Moreover, high activity of cathepsin D in the serum of squamous cell lung carcinoma patients was reduced after surgery (19), but no changes were observed in malignant lung tissue (20). As the role of procathepsin D in lung cancer is unclear, we decided to evaluate the hypothesis that procathepsin D and its activation peptide serve as growth factors for human lung cancer cells and that the active secretion of procathepsin D correlates with the procancerogenic properties of these cells.

Materials and Methods

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES, antibiotics, *Limulus* lysate test E-TOXATE, human cathepsin D and transferrin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and fetal calf serum (FBS) was from Hyclone Laboratories (Logan, UT, USA).

Monoclonal antibodies against activation peptide have been described previously (21). IgG was isolated from ascites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography. Monoclonal anti-cathepsin D antibodies were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Control MOPC-21 IgG antibody was purchased from Sigma Chemical Co. Mouse monoclonal anti-procathepsin antibody Ab-1 was purchased from Oncogene (San Diego, CA, USA).

The 44-amino-acid-long peptide corresponding to the activation peptide (AP) of procathepsin D was synthesized at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic. The purity of the AP was controlled using the following methods: HPLC, amino acid analysis and by mass spectrometry. The HPLC method showed the purity to be more than 95%. The amino acid analysis confirmed the

Correspondence to: Vaclav Vetvicka, Department of Pathology, School of Medicine, University of Louisville, 511 S. Floyd, MDR Bldg., Louisville, KY 40202, U.S.A. Tel: 502-852-1612, Fax: 502-852-1177, e-mail: vaclav.vetvicka@louisville.edu

Key Words: Lung cancer, procathepsin D.

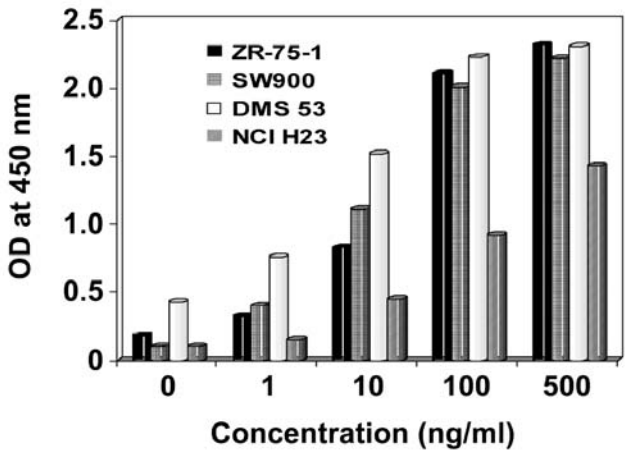

Figure 1. *Comparison of the growth of three human lung cancer cell lines SW900, DMS 53 and NCI H23 and the human breast cancer cell line ZR-75-1 in serum-free medium containing different concentrations of activation peptide.*

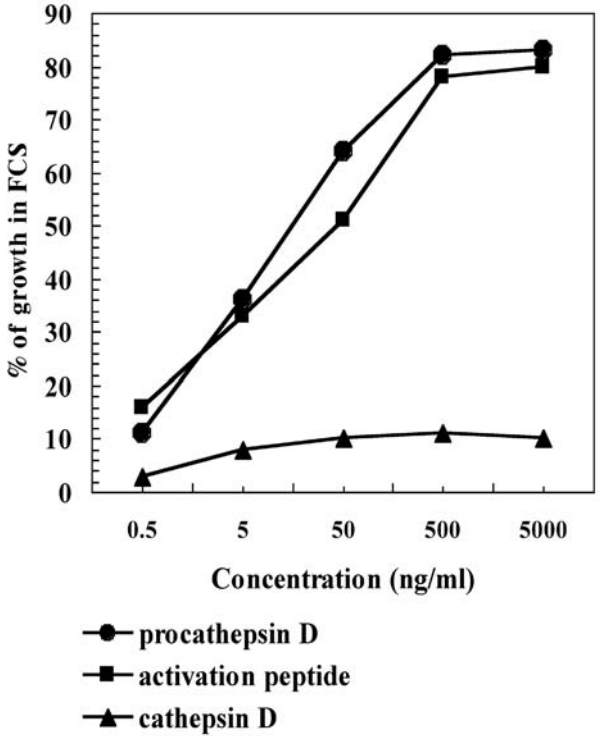

Figure 2. *The effect of various concentrations of either procathepsin D, activation peptide or cathepsin D on the human lung cancer cell line DMS-53 in serum-free medium. The growth in FCS-containing medium represented 100 percent.*

amino acid composition. Mass spectrometry results were in accordance with the proposed molecular structure. In addition to the techniques mentioned above, the purity of the peptide was also controlled by N–terminal sequencing using an automated system where the first 9 N–terminal amino acids were in agreement with the designed structure.

The human lung cancer cell lines DMZ-53, NCI H727, NCI H23, SW900 and human breast cancer cell line ZR-75-1 were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The cancer cell lines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, in plastic disposable tissue culture flasks at 37˚C in a 5%, CO_2/95% air incubator.

For growth experiments, cells were first incubated for 2 days in 0.1% FCS. The cells were harvested by centrifugation and washed six times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics and 10 μg/ml of human transferrin (6). The cells were seeded in 96-well tissue culture plates at a density of 5 x 10^4 cells/ml (150 μl/well) in the presence or absence of different concentrations of purified activation peptide in triplicate. After six days in culture, the proliferation was evaluated using a Biotrack ELISA system (Amersham Biosciences, Little Chalfont, UK). In all cell culture experiments, the cells were seeded in triplicates. We repeated our experiments using both charcoal-treated FBS and medium without phenol red with identical results.

Isolation of procathepsin D. Human procathepsin D was isolated from the culture supernatant of the human breast cancer cell line ZR-75-1, as described earlier (22). Briefly, a two-step procedure was used: first, immunoaffinity chromatography with anti-activation peptide antibodies attached to Protein A Sepharose. In the second step, FPC chromatography, using a Mono-Q column and 20 mM Tris-HCl (pH 7.2), was employed.

Western blotting. Ten times concentrated conditioned mediums from lung cancer cell lines were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were diluted 1:1 in Laemmli sample buffer and heated at 95 ˚C for 4 minutes (23). Electrophoresis of the denatured samples was carried out using 12% Tris-HCl ready precast gel (Biorad) at 200 V for 35 minutes at room temperature in 25 mM Tris-HCl, 190 mM glycine, 0.05% (w/v) SDS, pH 8.3 buffer. Proteins were then electro-transferred to nitrocellulose membrane (0.45 μm) (Biorad) at 100 V for one hour in 25mM Tris-HCl, 190 mM glycine, 20% (v/v) methanol. After blocking for one hour in 10 mM Tris-HCl, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5 (blocking buffer), the membrane was incubated with rabbit anti-procathepsin D polyclonal antibody (Oncogene) for one hour at room temperature. The membrane was then washed 3 times with blocking buffer and incubated for one hour with anti-rabbit Ig-alkaline phosphatase conjugate secondary antibody (Sigma) (1:10 000 in blocking buffer). After washing the membrane with blocking buffer (2 x) and blocking buffer without Tween 20 (2 x), procathepsin D-antibody complexes were detected using a NBT/BCIP alkaline phosphatase substrate kit.

Matrigel assay. For evaluation of the invasion across Matrigel layers, a commercial kit manufactured by Chemical International (Temecula, CA, USA) was used according to the manufacturer's instructions.

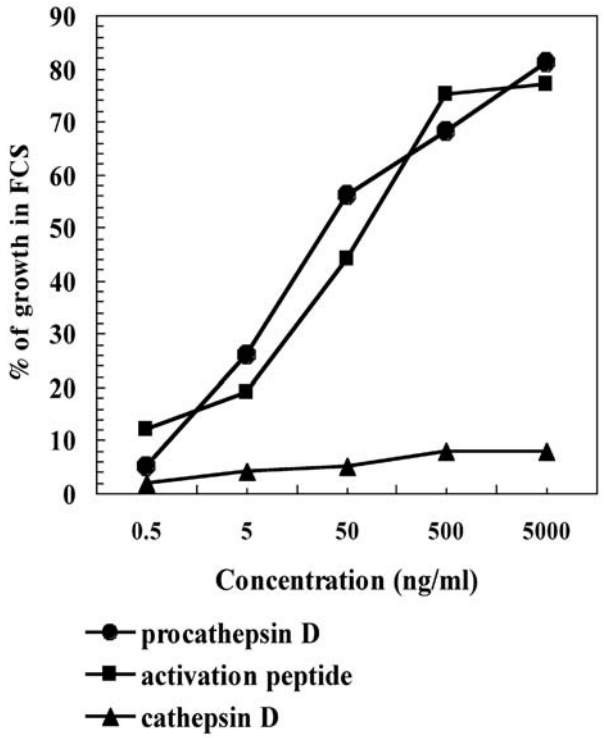

Figure 3. *The effect of various concentrations of either procathepsin D, activation peptide or cathepsin D on the human lung cancer cell line SW900 in serum-free medium. The growth in FCS-containing medium represented 100 percent.*

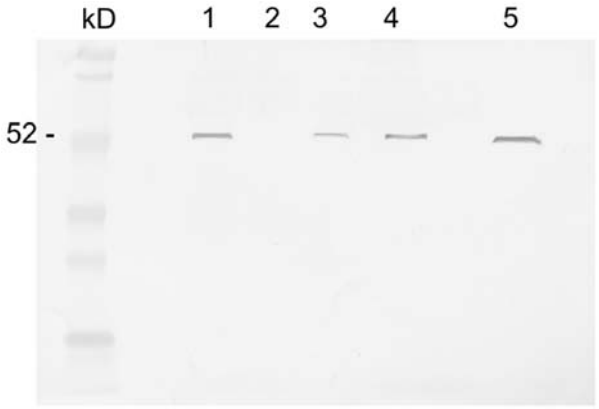

Figure 4. *Western blot of tissue culture media from different cell lines. 10x concentrated samples with anti-AP mAb were used for detection. Samples from left to right: NCI H727 (1), NCI H23 (2), DMS 53 (3), SW900 (4) and pCD (5). Procathepsin D was visualized using rabbit anti-procathepsin D polyclonal antibody.*

Results

Results showing the comparison of three lung cancer cell lines DMZ-53, SW900 and NCI H23 with the human breast cancer cell line ZR-75-1 are summarized in Figure 1. Both lung and breast lines showed the optimal dose of activation peptide as approximately 100 ng/ml; only in the case of the NCI H23 line, which in all instances was significantly lower compared to the other lines, was an increase in the proliferation shown even after 500 ng/ml concentration. The effects of entire procathepsin D, activation peptide and fully mature enzyme cathepsin D on the growth of two different lung cancer cell lines are shown on Figures 2 (DMZ-53) and 3 (SW900). The results showed that the effects of both procathepsin D and its activation peptide were virtually identical. Mature enzyme cathepsin D had absolutely no effects.

The data presented in these figures were obtained after 5 days of incubation. In our previous study on breast cancer cells we used a 7-day interval (6), but with faster growing prostate (2) and lung cancer cells we decided to shorten the tested interval. However, the data were in perfect agreement with data previously obtained with both breast and prostate cancer cells. Proliferation was measured by Biotrac ELISA assay measuring incorporation of BrdU.

Figure 4 demonstrates the differences in procathepsin D secretion into the tissue culture supernatant. Each of the tested cell lines showed a different level of procathepsin D secretion. Cell line NCI H727 was not used in the proliferation assay, as the growth of these cells in serum-free conditions did not allow us to test the effect of additional activation peptide.

For evaluation of the invasion across the Matrigel layer, we used a commercial kit. The results, summarized in Table I, clearly showed that the invasiveness across the membrane directly correlated with active secretion of procathepsin D, demonstrated by Western blotting (Figure 4).

Discussion

In recent years, an ever increasing amount of information has been collected on the important role of cathepsin D or procathepsin D in cancer development (for review see 16). Despite the original focus on enzymatically active cathepsin D, its role has never been satisfactorily documented and thus the subsequent research has been devoted to the biological and pathophysiological role of procathepsin D. After the original observation of procathepsin D secretion in breast cancer (3), subsequent studies demonstrated the more general involvement of procathepsin D in at least nine additional types of cancer. However, only very limited attention has been focused on lung cancer. Therefore, we decided to test the hypothesis that procathepsin D might act towards lung cancer-derived cells as an autocrine mitogen.

Table I. *Invasiveness through Matrigel membrane.*

Cell Line	Invasiveness	Secretion of Procathepsin D
SW900	+ +	+ +
DMS-53	+ +	+
NCI H23	-	-
NCI H727	+ +	+ +

The data presented in this report show that human procathepsin D has a considerable proliferative activity towards human lung cancer cell lines. Similarly to breast and prostate cell lines, procathepsin D exhibits its growth-stimulating activity even towards lines which do not spontaneously secrete procathepsin D (2,24). Using the activation peptide of the procathepsin D molecule, we found similar results as with the intact procathepsin D.

Experiments using procathepsin D, the enzymatically inactive precursor of cathepsin D, raise a possibility that the mature enzyme cathepsin D might be formed in the culture medium during the experiments. Secreted or added procathepsin D might be activated by the acidic extracellular conditions and subsequently degrade growth inhibitors and/or extracellular matrix. In addition, there are some studies showing direct growth factor-like effects of the fully mature enzyme cathepsin D (25). Subsequent study using mutated cathepsin D indicated that the proteolytic activity was not involved in a cancer-promoting or mitogenic function of procathepsin D in certain cancer tissues (26). Our experiments, showing no growth-promoting effects of active cathepsin D (Figures 2 and 3) in lung cancer as well as in breast (7) and prostate cancer models (27), demonstrated that cathepsin D had very little, if any, influence on cancer growth. In addition, the use of pepstatin A, a strong inhibitor of cathepsin D, with Ki at the picomolar level, showed no inhibition of cancer growth (27). The specificity of the experimental design was further demonstrated by preincubation of procathepsin D with two-different anti-procathepsin or anti-activation peptide antibodies coupled with Protein A Sepharose. The resulting solution completely lost its growth-potentiating activity (data not shown).

The data presented in this study are the first direct observations of the biological effects of procathepsin D on lung cancer cells. In addition to the role as a growth factor, procathepsin D might also be involved more directly in invasiveness, since the level of procathepsin D secretion closely correlated with invasiveness through the Matrigel membrane.

Based both on published data and on our own experiments, we have proposed the following model of the function of procathepsin D in cancer. Overexpressed procathepsin D escapes normal intracellular targeting pathways and is released from the cancer cells. Once secreted in substantial quantities, the well-documented uptake by mannose-6-phosphate receptors (28) cannot compensate for this extra flow of procathepsin and subsequently procathepsin D starts to accumulate. The active peptide part of the procathepsin D molecule interacts with a yet unidentified membrane receptor, yielding a release of signal/s resulting in accelerating division of parental cancer cells. Subsequently, the newly formed cancer cells secrete more procathepsin D in an autocrine loop (16).

The data gathered in this paper show that the biological action of procathepsin D is much more general than was originally suggested. Animal studies showing that immunization with the activation peptide resulted in significant suppression of implanted cancer cell growth (16) suggest that inhibition of procathepsin D secretion may be used in cancer treatment and prevention.

Acknowledgements

The work was supported by a research grant from the Kentucky Lung Cancer Fund, U.S.A.

References

1 Westley BR and May FEB: Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells. Nucleid Acid Res *15*: 3773-3786, 1987.

2 Vetvicka V, Vetvickova J and Fusek M: Effect of procathepsin D and its activation peptide on prostate cancer cells. Cancer Lett *129*: 55-59, 1998.

3 Vignon F, Capony F, Chambon M, Freiss G, Garcia M and Rochefort H: Autocrine growth stimulation of the MCF 7 breast cancer cells by the estrogen-regulated 52K protein. Endocrinology *118*: 1537-1545, 1986.

4 Rochefort H: Cathepsin D in breast cancer: A tissue marker associated with metastasis. Eur J Cancer *28A*: 1780-1783, 1992.

5 Garcia D, Dercoq Q, Pujol P and Rochefort H: Overexpression of transfected cathepsin D in transformed cells increases their malignant phenotype and metastatic potency. Oncogene *5*: 1809-1814, 1990.

6 Vetvicka V, Vetvickova J and Fusek M: Effect of human procathepsin D on proliferation of human cell lines. Cancer Lett *79*: 131-135, 1994.

7 Fusek M and Vetvicka V: Mitogenic function of human procathepsin D - role of the activation peptide. Biochem J *303*: 775-780, 1994.

8 Taylor DD and Gercel-Taylor C: Tumor-reactive immunoglobulins in ovarian cancer: diagnostic and therapeutic significance? Oncology Reports *5*: 1519-1524, 1998.

9 Strojan P, Budihna M, Smid L, Vrhovec I and Skrk J: Cathepsin D in tissue and serum of patients with squamous cell carcinoma of the head and neck. Cancer Letters *130*: 49-56, 1998.

10 Nazeer T, Church K, Amato C, Ambros RA, Rosano TG, Malfetano JH and Ross JS: Comparative quantitative immunohistochemical and immunoradiometric determinations of cathepsin D in endometrial adenocarcinoma: predictors of tumor aggressiveness. Mod Pathol *7*: 469-474, 1994.
11 Huet G, Zerimech F, Dieu M-C, Hemon B, Gerard G, Balduyck M, Janin A, Lafyatis R, Degand P, Dieu MC, Janin A and Lafyatis R: The state of differentiation of HT-29 colon carcinoma cells alters the secretion of cathepsin D and of plasminogen activator. Int J Cancer *57*: 875-882, 1994.
12 Ferrandina G, Scambia G, Panici P, Alamdori G, Paludetti G, Cadoni G, Distefano N, Maurizi M and Mancuso S: Cathepsin D in primary squamous laryngeal tumors: correlation with clinico-pathological parameters and receptor status. Cancer Lett *67*: 133-138, 1992
13 Chambon M, Rebillard X, Rochefort H, Brouillet JP, Baldet P, Guiter J and Maudelonde T: Cathepsin D cytosolic assay and immunohistochemical quantification in human prostate tumors. Prostate *24*: 320-325, 1994.
14 Tedone T, Correale M, Barbadossa G, Casavola V, Paradiso A and Reshkin SJ: Release of the aspartyl protease cathepsin D is associated with and facilitates human breast cancer cell invasion. FASEB J *11*: 785-792, 1997.
15 Johnson MD, Torri JA, Lippman ME and Dickson RB: The role of cathepsin D in the invasiveness of human breast cancer cells. Cancer Res *53*: 873-877, 1993.
16 Vetvicka V, Benes P and Fusek M: Procathepsin D in breast cancer: What do we know? Effects of ribozymes and other inhibitors. Cancer Gene Therapy *9*: 854-863, 2002.
17 Chyczewski L, Plonski A, Chyczewska E, Furman M, Ostrowska H, Niklinski J and Kozlowski M: Activity and tissue localization of cathepsin D in non small cell lung cancer. Roc Akad Medyc *42*: 217-229, 1997.
18 ZhixinW and Xiaoyan Z: Expression and prognostic relation of cathepsin D in non-small lung cancer tissues and lymph nodes. Chin J Tuberc Respir Dis *21*: 164-166, 1998.
19 Wozniak A, Drewa T, Rozwodowska M, Drewa G, Lambrecht W and Wisniewska I: Activity of some lysosomal enzymes in serum and in tumors of patients with squamous cell lung carcinoma. Neoplasma *49*: 10-15, 2002.
20 Ledakis P, Tester WT, Rosenberg N, Romero-Fishmann D, Daskal I and Lah TT: Cathepsins D, B, and L in malignant human lung tissue. Clin Cancer Res *2*: 561-568, 1996.
21 Vetvicka V, Vetvickova J and Fusek M: Anti-human procathepsin D activation peptide antibodies inhibit breast cancer development. Breast Cancer Res Treat *57*: 261-269, 1999.
22 Koelsch G, Metcalf P, Vetvicka V and Fusek M: Human procathepsin D: three-dimensional model and isolation, *In*: Aspartic Proteinases: Structure, Function, Biology, and Biomedical Implications (Takahashi K ed.). New York, Plenum Press. 1995, pp. 273-278.
23 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *277*: 680-585, 1970.
24 Vetvicka V, Vetvickova J, Hilgert I, Voburka Z and Fusek M: Analysis of the interaction of procathepsin D activation peptide with breast cancer cells. Int J Cancer *73*: 403-409, 1997.
25 Rochefort H and Capony F: Alterations of cathepsin D in human breast cancer. *In*: Proteolysis and Protein Turnover (Bond JS Barren AJ, eds). London, Portland Press. 1993, pp. 233-238.
26 Glondu M, Coopman P, Laurent-Matha V, Garcia M, Rochefort H and Liaudet-Coopman E: A mutated cathepsin D devoid of its catalytic activity stimulates the growth of cancer cells. Oncogene *20*: 6920-6929, 2001.
27 Vetvicka V, Vetvickova J and Fusek M: Role of procathepsin D activation peptide in prostate cancer growth. Prostate *44*: 1-7, 2001.
28 Dahms NM, Lobel P and Kornfeld S: Mannose-6-phosphate receptorts and lysosomal enzyme targeting. J Biol Chem *264*: 12115-12118, 1989.

Received November 14, 2003
Revised January 19, 2004
Accepted March 10, 2004