ORIGINAL ARTICLE

TJG
The Turkish Journal of Gastroenterology

Differential response of gastric carcinoma MKN-45 and 23132/87 cells to H_2O_2 exposure

Salih GENCER[1], Meliha Burcu IRMAK YAZICIOĞLU[2, 3]

Departments of [1]Genetics and Bioengineering, [3]Biology, Fatih University Faculty of Arts and Science, İstanbul
Department of [2]Molecular Biology and Genetics (Present), Haliç University Faculty of Arts and Science, İstanbul

Background/aims: *Reactive oxygen species are involved in tumor progression but how they function is not well understood. In this study, we investigated and compared the effects of hydrogen peroxide on the survival, apoptosis, accumulation of oxidative stress, and matrix metalloprotein-7 gene expression on human gastric carcinoma MKN-45 and 23132/87 cells.* ***Methods:*** *The cell lines were exposed to hydrogen peroxide in a dose- and time-dependent manner. The surviving cells were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The oxidative stress was analyzed by an oxidative stress marker, 2,7-dichlorofluorescein diacetate, under fluorescence microscope. The effect of oxidative stress on the apoptotic behavior, caspase-3 activity, and matrix metalloprotein-7 gene expressions of the cell lines were determined, respectively, by TUNEL, caspase-3 activity assay, and quantitative real-time polymerase chain reaction.* ***Results:*** *Exposure of the gastric cancer cells to oxidative stress resulted in dose- and time-dependent decrease in the survival of the cell lines. While MKN-45 cells had oxidative stress, increased apoptosis with no caspase-3 activity, and increased matrix metalloprotein-7 expression following hydrogen peroxide exposure, 23132/87 cells showed none, with internal oxidative stress accumulation prior to hydrogen peroxide exposure.* ***Conclusions:*** *The two gastric cancer cell lines responded differently to oxidative stress conditions, and unlike in 23132/87 cells, matrix metalloprotein-7 gene expression was shown to be affected by external hydrogen peroxide in MKN-45 cells.*

Key words: Hydrogen peroxide, oxidative stress, apoptosis, MMP-7, human gastric carcinoma cells

Gastrik kanseri MKN-45 ve 23132/87 hücrelerinin H_2O_2 muamelesine farklı tepkileri

Amaç: *Reaktif oksijen türleri tümör ilerlemesinde yer alır ancak bu işlevi nasıl yerine getirdikleri çok iyi anlaşılamamıştır. Bu çalışmada biz, hidrojen peroksitin insan gastrik kanser hücreleri MKN-45 ve 23132/87'nin yaşama, apoptozis, oksidatif stres biriktirme ve matriks metalloproteinaz 7 gen ekspresyonu üzerindeki etkilerini araştırıp karşılaştırdık.* ***Yöntem:*** *Hücre hatları doz ve zaman ayarlı olarak hidrojen peroksite maruz bırakıldı. Yaşayan hücreler 3-(4,5-dimethylthiazoil-2-yl)-2,5-diphenyltenrazolium bromide tahlili ile saptandı. Bu hücrelerde oksidatif stres bir oksidatif stres markırı olan 2,7-dichlorofluorescein diasetat ile florasan mikroskoupu altında analiz edildi. Hücrelerde, oksidatif stresin apoptotik davranış, kaspaz 3 aktivitesi ve matriks metalloproteinaz 7 gen ekspresyonuna etkisi sırası ile TUNEL, kaspaz 3 aktivite tahlili ve kantitatif RT-PCR ile ortaya çıkarıldı.* ***Bulgular:*** *Gastrik kanser hücrelerinin oksidatif strese maruz bırakılması ile doz ve zaman bağımlı olarak yaşamlarında azalma saptandı. MKN-45 hücresi hidrojen peroksit muamelesi sonucu oksidatif stres barındırıp, kaspaz-3 aktivitesi olmadan artan bir apoptozis ve matriks metalloproteinaz 7 ekspresyonunda artış gösterirken, 23132/87 hücreleri bunların hiçbirini göstermeden hidrojen peroksit muamelesi öncesi internal bir oksidatif stres akümülasyonu sergiledi.* ***Sonuç:*** *İki gastrik kanseri hücre hattı oksidatif stres kondüsyonlarına farklı tepkiler verdi, 23132/87 hücrelerinin aksine, MKN-45 hücrelerinde matriks metalloproteinaz 7 gen ekspresyonunun eksternal hidrojen peroksit ile etkilendiği gösterildi.*

Anahtar kelimeler: Hidrojen peroksit, oksidatif stress, apoptozis, MMP-7, insan gastric karsinoma hücreleri

INTRODUCTION

Oxidative damage, mediated by reactive oxygen species (ROS), has been implicated as a major cause of cellular injuries in a vast variety of clinical abnormalities, including cancer, diabetes, aging,

Address for correspondence: Meliha Burcu IRMAK YAZICIOĞLU
Molecular Biology and Genetics, Faculty of Arts and Sciences,
Halic University, Kaptanpaşa, Darülaceze Str., No:14,
Okmeydanı, İstanbul, 34383, Turkey
Phone: + 90 212 220 96 96 Ext: 126 • Fax: + 90 212 210 46 08
E-mail: burcuyazicioglu@halic.edu.tr

Manuscript received: 07.06.2010 **Accepted:** 11.12.2010

Turk J Gastroenterol 2011; 22 (2): 145-151
doi: 10.4318/tjg.2011.0183

cardiovascular disease, and neurodegenerative disorders (1). Oxidative stress results when the balance between the production of ROS overrides the antioxidant capability of the target cell; oxidative damage from the interaction of reactive oxygen with critical cellular macromolecules may occur (2). It may play a role in carcinogenesis through its numerous effects on the cells, including damage to protein and lipid, and DNA consequently changes in membrane structure and function (3-5) and gene expression (6-8). This in turn leads to alterations in cell turnover and enhanced cell death, and subsequently, the accumulation of oxidative damage causes formation of cancer (9). Previously, elevated oxidative status has been found in many types of cancer cells, which contribute to carcinogenesis (10-13).

Gastric cancer (GC) is the seventh most common cancer and the second most common cause of cancer-related death worldwide (14). The gastric epithelium is continuously exposed to toxic ROS within the gastric lumen due to ingested food and cigarette smoke and inflammation due to *Helicobacter pylori* infection. The dynamic balance between cell proliferation and apoptosis is essential for maintaining mucosal homeostasis. Decreased apoptosis as well as increased proliferation may favor the carcinogenic process. Prolonged survival of abnormal cells can support the accumulation of sequential genetic mutations, changes in gene expression profiles and protein structure and function, which can result in tumor promotion (10,15-18).

Moreover, studies directed to matrix metalloproteins (MMP) analysis in tumor invasion and metastases have attracted the attention of scientists, since these proteins have the capability to degrade all the components of connective tissue, which is an acquired characteristic for a tumor cell. The studies on *MMP-7* in particular have attracted attention due to the fact that, being one of the MMPs involved in the majority of connective tissue destruction during invasion and metastasis of tumor, it is activated by nuclear β-catenin. In addition, it is well known that nuclear β-catenin is a hallmark of an active oncogenic Wnt pathway in gastric cancers (19). Recently, while the involvement of ROS signaling and the importance of MMP in tumor metastasis were highlighted (20), there is limited information in the literature related to this connection. To our knowledge, there is no work showing the effect of H_2O_2 exposure on *MMP-7* gene expression in gastric cancer cells.

Therefore, in this work, MKN-45 and 23132/87 cell lines were exploited to investigate and compare the effects of H_2O_2 exposure on survival, apoptosis, accumulation of oxidative stress, and the quantitative expression of the *MMP-7* gene.

MATERIALS AND METHODS

Cell Culture

Human gastric adenocarcinoma cell lines, MKN-45 (DSMZ ACC409) and 23132/87 (DSMZ ACC201), were purchased from DSMZ. The cells were cultured in a standard RPMI-1640 (Biochrom) medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin and streptomycin (10000µg/mL, Biochrom) in a humidified chamber at 37° C in the presence of 5% CO_2.

H_2O_2 Treatment of the Cell Lines

The cells were plated at a density of $3x10^3$–$5x10^3$ cells per well and cultured in the standard medium overnight. The medium was aspirated and washed with phosphate-buffered saline (PBS); the cells were starved in the culture medium supplemented with 0.01% FBS overnight, and they were treated with indicated concentrations of H_2O_2 ranging from 50-1000 µM for the indicated time points. The control cells were only starved and were not exposed to H_2O_2.

Determination of Cell Viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, which is used for cell proliferation and cytotoxicity measurement (21). Briefly, MTT (Sigma) reagent was prepared as 5 mg/ml in RPMI-1640 medium as a stock solution. The cell lines were cultured in 96-well culture plates for indicated times following indicated concentrations of H_2O_2 exposure. Then, after addition of MTT solution 10 µL/well (1:10, v/v), they were incubated for a further 4 hours (h). Finally, 50% DMF and 10% SDS were added as 100 µL/well as a stop solution (1:1, v/v), and optical density (OD) was measured on BioTek Power Wave XS microplate autoreader at 570 nm.

Determination of In-Situ Oxidative Stress

Oxidative stress in the cell lines was assessed using an oxidative stress marker, 2,7-dichlorofluorescein diacetate (DCFDA) (22). The nonfluorescent DCFH-DA (Sigma) is a cell-permeable compound that can enter into the cells, where it is deacetylated and entrapped as DCFH, the oxidation

of which by ROS produces a highly fluorescent product, DCF, which can be visualized under a fluorescent microscope. DCFDA is freshly prepared in 10 mM HEPES (pH 7.5), 10 mM glucose and 1µM DCFDA (dissolved in methanol) in PBS. The cells were treated with indicated concentrations of H_2O_2 ranging from 50-1000 µM for 6, 12, 24, and 48 h. After this time point, the medium was aspirated, washed two times with PBS, and the cells were incubated with DCFDA solution at 37°C for 15 minutes (min), and then they were washed two times with PBS and observed under fluorescent microscope (Axio-skop, Zeiss). The live cells that were under oxidative stress were counted in five 20x fields per culture (typically 70–100 cells/20x field), and the percentage of the cells under oxidative stress in each culture was calculated.

Determination of Apoptosis: TUNEL Assay

The apoptotic cells were visualized by terminal deoxytransferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) method (23). This assay was performed using In Situ Cell Death Detection kit (Roche), according to the manufacturer's recommendations. Briefly, the cells were seeded on autoclave-sterilized coverslips in 6-well plates and cultured overnight. The cells were treated with concentrations of H_2O_2 ranging from 50-200 µM for 24 h. They were washed with PBS two times and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, they were washed with PBS two times again and treated with TUNEL solution, and incubated at 37°C for 1 h. The apoptotic cells were observed under fluorescence microscopy (Axio-skop, Zeiss). Then, the apoptotic cells were counted in five 20x fields per culture (typically 70–100 cells/20x field), and the percentage of cells under oxidative stress in each culture was calculated.

Determination of Apoptosis: Caspase-3 Activity Assay

The level of apoptosis of cells was determined by caspase-3 activity using caspase-3 assay kit, Colorimetric (Sigma). The cells were seeded on autoclave-sterilized coverslips in 6-well plates. They were exposed to the indicated concentrations of H_2O_2 ranging from 50-1000 µM for 24 h. The total protein was determined by Bio-Rad protein assay and the activity of caspase-3 was analyzed following the instructor's manual.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated and cDNA was synthesized from the cell lines using RNA extraction kit (NucleoSpin RNA II, Macherey-Nagel) and cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Fermentas), respectively, according to the manufacturer's instructions. Sense 5'-TCCCGCGTCATAGAAATAATG-3' and antisense 5'-AGGAATGTCCCATACCCAAAG-3' primer pairs (451 bp) were used to investigate the expression of *MMP-7* (Gene accession number: AY795972). The *GAPDH* (Gene accession number: M33197) mRNA was also amplified in PCR reactions as an internal control using sense 5'-GACCTGCCGTCTAGAAAAAC-3' and antisense 5'-TTGAAGTCAGAGGAGACCAC-3' primer pairs (126 bp). The reactions were carried with 2 µl cDNA template in a total volume of 25 µl, containing 1X SYBR Green Supermix (Bio-Rad) with primers for *MMP-7* and *GAPDH* genes in Corbett Research RT-PCR machine. After initial denaturation at 95°C for 10 min, 40 cycles of 10 second (s) denaturation at 95°C, 25 s annealing at 57°C for *MMP-7* and 63°C for GAPDH, and 25 s extension at 72°C were carried out. Finally, melting analysis was performed in the temperature range of 55°C to 95°C to verify product homogeneity. RT-PCR reactions were carried out in triplicate for each sample as technical replicates. Each cDNA sample was tested in three different reactions with three technical replicates and negative controls. Three biological replications were performed for each transcript in order to determine if there were significant differences in the expressions at the different time points.

Statistical Analysis

All data were expressed as mean ±S.D. and evaluated using the t-test with one way analysis of variance (ANOVA). Differences were considered as statistically significant when $p<0.05$.

RESULTS

Cell Viability Analysis

In order to determine the effect of H_2O_2 on the survival of gastric carcinoma cells, a dose response curve was plotted. The antiproliferative effect of H_2O_2 on gastric carcinoma cell lines increased in a dose- and time-dependent manner. At H_2O_2 concentrations ranging from 50-200 µM, there was a 10-40% reduction in the survival of the cell lines, while at 500-1000 µM of H_2O_2, the cell death reached 70-100% (Figure 1).

Oxidative Stress

Gastric cancer cells, MKN-45 and 23132/87, showed a different oxidative stress profile following

H_2O_2 exposure in a dose- and time-dependent manner. As seen in Figure 2, 23132/87 cells had already accumulated ROS at 6 h due to starvation without any H_2O_2 exposure, and the accumulated ROS increased in a time-dependent manner without external H_2O_2 application. In addition, 50 μM exogenous H_2O_2 was enough to increase oxidative stress in these cells to about 70% following 12 h of incubation. On the other hand, due to starvation, the accumulated ROS were already at the levels of 70-100% at the other time points, which was not affected by further external stress. Unlike 23132/87, MKN-45 did not have any ROS accumulation prior to H_2O_2 exposure. There was a linear increase in oxidative stress in a dose- and time-dependent manner in viable MKN-45 cells, reaching 50% at 200 μM for 12 h of exposure (Figure 2).

Determination of Apoptosis

Apoptotic response of the cells to increasing concentrations of H_2O_2 (50-200 μM) at 24 h was analyzed by TUNEL assay (Figure 3a). Like oxidative stress analysis, the two cell lines gave different profiles of apoptosis. There was an increase in the percentage of apoptotic MKN-45 cells in a dose-dependent manner. 50 μM H_2O_2 was enough to induce apoptosis in about 40% of the cells. On the other hand, 23132/87 cells did not show any apoptotic response to any doses of H_2O_2. Contrary to the positive TUNEL signals, caspase-3 activation was not observed in MKN-45 cells upon H_2O_2 exposure (Figure 3b).

Quantitative RT-PCR Analysis

The expression of *MMP-7* gene in MKN-45 cells was up regulated 1.65, 2.61, and 1.93 times following 50, 100, and 200 μM H_2O_2 exposure for 12 h compared to the control (untreated), respectively. Conversely, 23132/87 cells did not express *MMP-7* gene and did not give any signal for *MMP-7* in the absence or presence of H_2O_2 following overnight incubation with 0.01% FBS (Figure 4a). The expression of MMP-7 gene was up regulated only 1.3 times following 100 μM H_2O_2 exposure for 24 h. The expression rate of this gene was the same as in the untreated cells following 50 μM and 200 μM H_2O_2 exposure for 24 h (Figure 4b).

DISCUSSION

Reactive oxygen species (ROS) are intricately involved in tumor progression through effects on proliferation, apoptosis and metastasis. However, how ROS work is not well understood (24). ROS

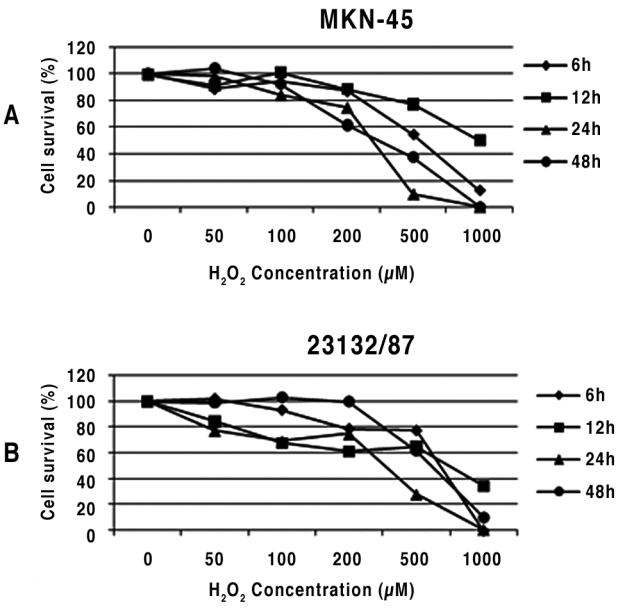

Figure 1. The effects of H_2O_2 exposure on the viability of MKN-45 (A) and 23132/87 (B) cells. Survival curves of the cells following exposure to varying concentration of H_2O_2 for 6, 12, 24, and 48 h were plotted. The viable cells were quantified by MTT assay.

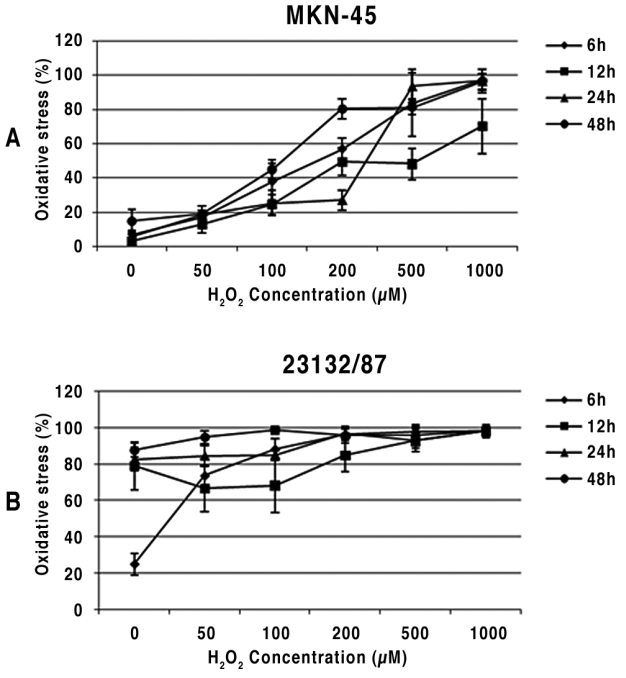

Figure 2. In-situ oxidative stress rate of H_2O_2-treated MKN-45 (A) and 23132/87 (B) cells. Oxidative stress in the cell lines was assessed using an oxidative stress marker, DCFDA. Error bars represent the average and standard deviations of three independent experiments.

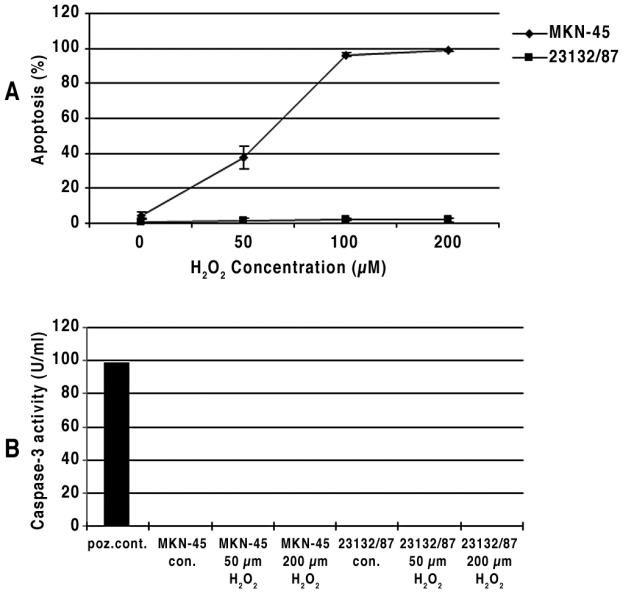

Figure 3. Apoptosis rate of H_2O_2-treated MKN-45 and 23132/87 cells for 24 h. Apoptosis was determined by TUNEL method (A). Caspase-3 activity of H_2O_2-treated MKN-45 and 23132/87 cells for 24 h (B). Error bars represent the average and standard deviations of three independent experiments.

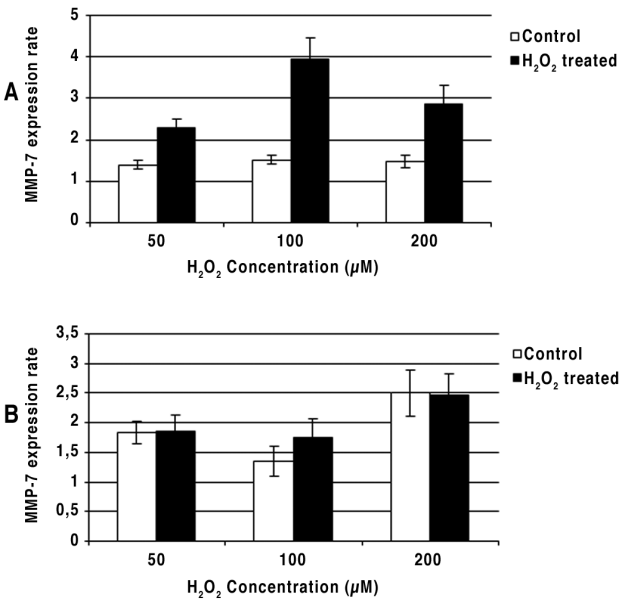

Figure 4. MMP-7 expression rate of H_2O_2-treated MKN-45 cells for 12 h (A) and 24 h (B). Expression rate was determined by quantitative RT-PCR. Error bars represent the average and standard deviations of three independent experiments.

can have effects on the cellular mechanisms involved in elimination of oxidative stress (10,25-27) and regulation of the molecules that play a role in the cell cycle (28-30). Subsequently, oxidative stress promotes cancer development. Despite increasing awareness about the importance of oxidative stress in gastric cancers, the effect of ROS on gastric carcinogenesis is still poorly understood (10). This study compares the response of the two gastric carcinoma cells, MKN-45 and 23132/87, to H_2O_2 exposure in terms of survival, apoptosis, accumulation of oxidative stress, and the quantitative expression of the *MMP-7* gene, which is involved in metastasis (31,32).

Our observations with the MKN-45 and 23132/87 cell lines demonstrated that in vitro exposure of H_2O_2 caused accumulation of oxidative stress in the former viable cells, while starvation without H_2O_2 treatment for more than 6 hours already resulted in the accumulation of oxidative stress in a high percentage of the latter cells (Figure 2b). The accumulation of ROS in the studied cell lines correlates with decreased cell survival. Most probably, MKN-45 and 23132/87 cells cannot cope with and consequently cannot eliminate external H_2O_2 or the metabolically derived oxidative stress, respectively.

There was a 1.65-, 2.61-, and 1.93-fold increase in *MMP-7* expression in MKN-45 cells upon exposure to 50, 100 and 200 µM H_2O_2, respectively, compared to the control cells, with 100 µM concentration being the most effective (Figure 4a). On the other hand, the 23132/87 cell line, which is studied for the first time in the literature in this study, did not have any *MMP-7* gene expression (data not shown). This cell line was already under stress prior to H_2O_2 treatment, most probably due to starvation of the cells. Strikingly, 12 hours of starvation was enough to accumulate oxidative stress in 80% of viable 23132/87 cells, pointing out that 23132/87 cells were even more prone to accumulation of stress since they most probably cannot cope with metabolically produced oxidative stress upon starvation. In addition, external H2O2 treatment did not induce a remarkable change in the accumulation of stress in these cells. Unlike 232132/87, it is obvious that MKN-45 did not accumulate an internal stress due to starvation. Contrary to 23132/87 cells, MKN-45 cells expressed the *MMP-7* gene; apparently, this may result in gaining increased invasive and metastatic characteristics of these cells. To our knowledge, this is the first work in the literature that revealed increased *MMP-7* gene expression in MKN-45 cells following H_2O_2 exposure.

The second, but probably the most challenging, of

our observations is that exogenous H_2O_2 induced apoptosis in the MKN-45 cell line, which was demonstrated by TUNEL assay, while the 23132/87 cell line did not show any apoptotic response under the same conditions even though it had ROS accumulation. The impairment of apoptotic programs in tumor development is an important process (33). Our data demonstrated that the apoptotic response of MKN-45 cells was caspase-3-independent, which is supported by the literature (34,35). In support of this, apoptosis in caspase-inhibited neuron cells was also reported (36). This apoptotic response of the MKN-45 cells is associated with the decreased cell survival and correlates with oxidative stress and the increased expression of the *MMP-7* gene, which is known to be one of the key molecules leading to enhanced proliferation, invasion and metastasis in some malignant tumor cells (37). There are several studies indicating up regulation of *MMP-7* in gastric cancer cells (38-40). It seems that H2O2 exposure in these apoptosis-sensitive cells induces apoptosis and simultaneously activates the expression of the *MMP-7* gene via activating some transcription factors that are known to be activated by H_2O_2 (41), resulting in over expression of *MMP-7*. A few data have been reported that, while triggering the apoptosis of tumor cells, proliferation of the tumor cells could also be induced through non-apoptotic signaling pathways (37). On the other hand, most probably, 23132/87 cells developed a resistance mechanism against apoptosis under oxidative stress conditions, which might give these cells a survival advantage and a more aggressive phenotype even in the absence of *MMP-7* expression. In conclusion, the two gastric carcinoma cell lines, MKN-45 and 23132/87, gave different apoptotic, oxidative stress and *MMP-7* gene expression responses to H_2O_2 exposure.

Acknowledgements: *This project was supported by the Scientific and Technical Research Council of Turkey (TUBITAK) under project number 105S352 (SBAG-K-110) and by the Scientific Research Fund of Fatih University under project number P50030703.*

REFERENCES

1. Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology 2000; 7: 153-63.
2. Oxidative stress: introduction. In: Sies H, ed. Oxidative stress: oxidants and antioxidants. San Diego, CA: Academic Press, 1991.
3. Breimer LH. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. Mol Carcinog 1990; 3: 188-97.
4. Storz G, Polla BS. Transcriptional regulators of oxidative stress-inducible genes in prokaryotes and eukaryotes. Exper Suppl Basel 1996; 77: 239-54.
5. Schenk H. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-icB and AP-1. Proc Natl Acad Sci USA 1994; 91: 1672-6.
6. Dasari A, Bartholomew JN, Volonte D, et al. Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/sp1-mediated activation of two GC-Rich promoter elements. Cancer Res 2006; 66: 10805-14.
7. Carnesecchi S, Carpentier JL, Foti M, et al. Insulin-induced vascular endothelial growth factor expression is mediated by the NADPH oxidase NOX3. Exp Cell Res 2006; 312: 3413-24.
8. Brown-Bryan TA, Leoh LS, Ganapathy V, et al. Alternative splicing and caspase-mediated cleavage generate antagonistic variants of the stress oncoprotein LEDGF/p75. Mol Cancer Res 2008; 6: 1293-307.
9. Klaunig JE, Xu Y, lsenberg JS, et al. The role of oxidative stress in chemical carcinogenesis. Environ Health Perspect 1998; 106 (Suppl): 289-95.
10. Smoot DT, Elliott TB, Verspaget HW, et al. Influence of *Helicobacter pylori* on reactive oxygen-induced gastric epithelial cell injury. Carcinogenesis 2000; 21: 2091-5.
11. Ambrosone CB. Oxidants and antioxidants in breast cancer. Antioxid Redox Signal 2000; 2: 903-17.
12. Emerit I. Reactive oxygen species, chromosome mutation, and cancer: possible role of clastogenic factors in carcinogenesis. Free Radic Biol Med 1994; 16: 99-109.
13. Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 1999; 49: 91-102.
14. Farinati F, Cardin R, Cassaro M, et al. Helicobacter pylori, inflammation, oxidative damage and gastric cancer: a morphological, biological and molecular pathway. Eur J Cancer Prev 2008; 17: 195-200.
15. Karam SM. Cellular origin of gastric cancer. Ann N Y Acad Sci 2008; 1138: 162-8.
16. Yoshida N, Yoshikawa T, Iinuma S, et al. Rebamipide protects against activation of neutrophils by *Helicobacter pylori*. Dig Dis Sci 1996; 41: 1139-44.
17. Suzuki M, Nakamura M, Mori M, et al. Lansoprazole inhibits oxygen-derived free radical production from neutrophils activated by *Helicobacter pylori*. J Clin Gastroenterol 1995; 20: S93-S96.
18. Davies GR, Banatvala N, Collins CE, et al. Relationship between infective load of *Helicobacter pylori* and reactive oxygen metabolite production in antral mucosa. Scand J Gastroenterol 1994; 29: 419-24.
19. Kolligs FT, Bommer G, Goke B. Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. Digestion 2002; 66: 131-4.
20. Wu WS. The signaling mechanism of ROS in tumor progression. Cancer Metastasis Rev 2006; 25: 695-705.
21. Heeg K, Reimann J, Kabelitz D, et al. A rapid colorimetric assay for the determination of IL-2-producing helper T cell frequencies. J Immunol Methods 1985; 77: 237-46.
22. Bass A, Parce JW, Dechatelet LR, et al. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983; 130: 1910-7.

23. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493-501.
24. Wang X, Wang J, Lin S, et al. Sp1 is involved in H2O2-induced PUMA gene expression and apoptosis in colorectal cancer cells. Exp Clin Cancer Res 2008; 24: 27-44.
25. Petit PX, Susin SA, Zamzami N, et al. Mitochondria and programmed cell death: back to the future. FEBS Lett 1996; 396: 7-13.
26. Marchetti P, Castedo M, Susin SA, et al. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 1996; 184: 1155-60.
27. Castedo M, Macho A, Zamzami N, et al. Mitochondrial perturbations de?ne lymphocytes undergoing apoptotic depletion in vivo. Eur J Immunol 1995; 25: 3277-84.
28. Havens CG, Ho H, Yoshioka N, et al. Regulation of late G1/S phase transition and APCCdh1 by reactive oxygen. Mol Cell Biol 2006; 26: 4701-11.
29. Bragado P, Armesilla A, Silva A, et al. Apoptosis by cisplatin requires p53 mediated p38alpha MAPK activation through ROS generation. Apoptosis 2007; 12: 1733-42.
30. Macip S, Kosoy A, Lee SW, et al. Oxidative stress induces a prolonged but reversible arrest in p53-null cancer cells, involving a Chk1-dependent G2 checkpoint. Oncogene 2006; 25: 6037-47.
31. Miller JJ, Summers MK, Hansen DV, et al. Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. Genes Dev 2006; 20: 2410-20.
32. Mylona E, Kapranou A, Mavrommatis J, et al. The multifunctional role of the immunohistochemical expression of *MMP-7* in invasive breast cancer. APMIS 2005; 113: 246-55.
33. Zhivotovsky B, Orrenius S. Carcinogenesis and apoptosis: paradigms and paradoxes. Carcinogenesis 2006; 27: 1939-45.
34. Wu ZQ, Zhang R, Chao C, et al. Histone deacetylase inhibitor trichostatin A induced caspase-independent apoptosis in human gastric cancer cell. Chin Med J (Engl) 2007; 120: 2112-8.
35. Kasuga T, Tabuchi T, Shirato K, et al. Caspase-independent cell death revealed in human gastric cancer cell lines, MKN45 and KATO III, treated with phenoxazine derivatives. Oncol Rep 2007; 17: 409-15.
36. Volbracht C, Leist M, Kolb SA, et al. Apoptosis in caspase-inhibited neurons. Mol Med 2001; 7: 36-48.
37. Zhou DH, Trauzold A, Röder C, et al. The potential molecular mechanism of overexpression of uPA, IL-8, *MMP-7* and MMP-9 induced by TRAIL in pancreatic cancer cell. Hepatobiliary Pancreat Dis Int 2008; 7: 201-9.
38. Wroblewski LE, Noble PJ, Pagliocca A, et al. Stimulation of *MMP-7* (matrilysin) by *Helicobacter pylori* in human gastric epithelial cells: role in epithelial cell migration. J Cell Sci 2003; 116: 3017-26.
39. Hofman VJ, Moreilhon C, Brest PD, et al. Gene expression profiling in human gastric mucosa infected with *Helicobacter pylori*. Mod Pathol 2007; 20: 974-89.
40. Safranek J, Holubec L Jr, Topolcan O, et al. Expression of mRNA *MMP-7* and mRNA TIMP-1 in non-small cell lung cancer. Anticancer Res 2007; 27: 2953-6.
41. Majumdar S, Lamothe B, Aggarwal BB. Thalidomide suppresses NF-kappa B activation induced by TNF and H_2O_2, but not that activated by ceramide, lipopolysaccharides, or phorbol ester. J Immunol 2002; 168: 2644-51.