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Electromagnetic Fields at Mobile Phone Frequency Induce Apoptosis and Inactivation of the Multi-chaperone Complex in Human Epidermoid Cancer Cells

MICHELE CARAGLIA,[1,2]** MONICA MARRA,[1] FABRIZIO MANCINELLI,[1] GUGLIELMO D'AMBROSIO,[3] RITA MASSA,[3] ANTONIO GIORDANO,[4] ALFREDO BUDILLON,[2] ALBERTO ABBRUZZESE,[1]* AND ETTORE BISMUTO[1]

[1]*Department of Biochemistry and Biophysics, Second University of Naples, Italy*
[2]*Experimental Pharmacology Unit, National Institute of Tumours ''Fondazione G. Pascale'' of Naples, Italy*
[3]*ICEmB at Department of Electronic and Telecommunication Engineering, University ''Federico II'' of Naples, Naples, Italy*
[4]*Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania and Department of Human Pathology and Oncology University of Siena, Italy*

The exposure to non-thermal microwave electromagnetic field (MW-EMF) at 1.95 MHz, a frequency used in mobile communication, affects the refolding kinetics of eukaryotic proteins (Mancinelli et al., 2004). On these basis we have evaluated the in vivo effect of MW-EMF in human epidermoid cancer KB cells. We have found that MW-EMF induces time-dependent apoptosis (45% after 3 h) that is paralleled by an about 2.5-fold decrease of the expression of ras and Raf-1 and of the activity of ras and Erk-1/2. Although also the expression of Akt was reduced its activity was unchanged likely as a consequence of the increased expression of its upstream activator PI3K. In the same experimental conditions an about 2.5-fold increase of the ubiquitination of ras and Raf-1 was also found and the addition for 12 h of proteasome inhibitor lactacystin at 10 μM caused an accumulation of the ubiquitinated isoforms of ras and Raf-1 and counteracted the effects of MW-EMF on ras and Raf-1 expression suggesting an increased proteasome-dependent degradation induced by MW-EMF. The exposure of KB cells to MW-EMF induced a differential activation of stress-dependent pathway with an increase of JNK-1 activity and HSP70 and 27 expression and with a reduction of p38 kinase activity and HSP90 expression. The overexpression of HSP90 induced by transfection of KB cells with a plasmid encoding for the factor completely antagonized the apoptosis and the inactivation of the ras → Erk-dependent survival signal induced by MW-EMF. Conversely, the inhibition of Erk activity induced by 12 h exposure to 10 mM Mek-1 inhibitor U0126 antagonized the effects induced by HSP90 transfection on apoptosis caused by MW-EMF. In conclusion, these results demonstrate for the first time that MW-EMF induces apoptosis through the inactivation of the ras → Erk survival signaling due to enhanced degradation of ras and Raf-1 determined by decreased expression of HSP90 and the consequent increase of proteasome dependent degradation. J. Cell. Physiol. 204: 539–548, 2005. © 2005 Wiley-Liss, Inc.

Mobile phone use has dramatically increased with reducing costs and industrial sources suggest that there will be over one billion users worldwide by 2005 (Repacholi, 2001). As a consequence there is an increasing public interest about health hazard due to radiofrequency fields exposure (Hyland, 2000; Laurence et al., 2000; Adair, 2002, 2003). Although few epidemiological studies are available about radiofrequency exposure and development of specific pathologies (Goldsmith, 1995; Rothman, 2000), suggestive evidences for a health risk have been presented in the recent literature (Repacholi et al., 1997; French et al., 2000; Kalns et al., 2000; Youbicier-Simo and Bastide, 2000; Higashikubo et al., 2001; Zook and Simmens, 2001; Leszczynski et al., 2002; Mashevich et al., 2003). On the other hand, the use of microwaves is emerging as an additional non-invasive approach for the therapy of human neoplasms, also taking advantage from the generation of shock responses and apoptosis in human cancer cells (Maeda et al., 2004). Non-thermal effects of microwave exposure at frequencies of mobile phone, that is 800–1800 MHz, have been described in several reports. We have recently demonstrated that microwaves generated by an electromagnetic field (MW-EMF) can affect the three dimensional structure of eukaryotic proteins also suggesting possible biological effects in living cells (Mancinelli et al., 2004). Moreover, it has been demonstrated that prolonged exposure to low-intensity microwaves fields can induce heat-shock responses,

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*Correspondence to: Alberto Abbruzzese, Dipartimento di Biochimica e Biofisica, Seconda Università di Napoli, Via Costantinopoli 16, 80138 Napoli, Italy. E-mail: Alberto.Abbruzzese@unina2.it

**Correspondence to: Michele Caraglia, UNITA di Farmacologia Sperimentale, Istituto Nazionale dei Tumori "Fondaz. G. Pascale" di Napoli, Via M. Semmola, 80131 Napoli, Italy.
E-mail: michele.caraglia@fondazionepascale.it

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suggesting damage to cellular proteins. The heat-shock proteins (HSPs) could be involved as molecular chaperones to rescue damaged proteins (De Pomerai et al., 2000; French et al., 2000; Hyland, 2000; Leszczynski et al., 2002). The heat shock response was first described in 1962 (Ritossa, 1962), and HSPs are named for their increased synthesis after heat shock that is contrary to the reduced synthesis of most cellular proteins under these conditions. In addition to heat, these proteins are modulated by nutrient deprivation, and oxidative and other stresses where protein denaturation might otherwise occur (Morimoto et al., 1997; Hartl and Hayer-Hartl, 2002). Many HSPs form multimolecular complexes that act as molecular chaperones and bind other proteins, denoted as client proteins (Goetz et al., 2003). These complexes play a regulatory role in the fate of proteins in several different ways including: folding of proteins in the cytosol, endoplasmic reticulum and mitochondria; intracellular transport of proteins; repair or degradation of proteins partially denatured by exposure to various environmental stresses; control of regulatory proteins; and refolding of misfolded proteins (Morimoto et al., 1997; Hartl and Hayer-Hartl, 2002). Mammalian HSPs have been classified into several families according to their molecular size: HSP90, HSP70, HSP60, and HSP40, and the small HSPs such as HSP27 (Morimoto et al., 1997; Hartl and Hayer-Hartl, 2002). Within the different HSPs the isoform with 90 kDa MW (HSP90) acts in concert with other chaperones and partners (HSP70, p23, HOP, and p50/Cdc) to provide maturation and folding, as well as trafficking and function of their client proteins (c-Raf, ras, ErbB-2, mitogen extracellular signal regulated kinase, Mek, epidermal growth factor receptor, and steroid receptors) through the formation of the HSP90/multi-chaperone complex (Blagosklonny, 2002). Several protein kinases, including Raf-1, ErbB-2, and Bcr-Abl depend upon the HSP90/multi-chaperone for proper function and stability and this is likely the way by which HSP90/multi-chaperone is involved in the regulation of apoptotic processes (Creagh et al., 2000; Jolly and Morimoto, 2000; Richter and Buchner, 2001; Young et al., 2001). In fact, the HSP90 client proteins Raf-1 and MEK are components of the ras → extracellular signal regulated kinase (Erk)-dependent signal transduction pathway that is involved in regulation of both proliferation and apoptosis (Caraglia et al., 1999). In details, Raf-1, stimulated after steric interaction with Ras, phosphorylates and activates a MKK whose main component is Mek-1. Mek-1 phosphorylates the MAPKs Erk 1 and 2 that translocate to the nucleus and phosphorylate gene transactivators, such as the serum response factor-1 (Garrington and Johnson, 1999; Widmann et al., 1999). A second important anti-apoptotic pathway involves signaling via Akt/PKB, another HSP90 client (Zhou et al., 2000; von Gise et al., 2001). In fact, it has been demonstrated that Akt can be activated concomitantly or independently from Ras → ERK-1/2 signaling by growth factors (Kuo et al., 2001; Liu et al., 2001; Mitsui et al., 2001). Additionally, it has been reported that PC12 cells display a protective anti-apoptotic pathway in response to hypoxic stimuli (Alvarez-Tejado et al., 2001). The protection from apoptosis by Akt could be due to the regulation of mitochondrial physiology since Akt is involved in the regulation of bcl-related proteins such as Mcl-1 (Kuo et al., 2001). However, the requirement of Akt for the protection from apoptotic events is highly variable depending upon the experimental model used (Chaudhary and Hruska, 2001; Liu et al., 2001; Mitsui et al., 2001). In this view, we have previously demonstrated that human epidermoid cancer KB cells can undergo to apoptosis through the triggering of a stress response that leads to the activation of NH2-terminal Jun kinase-1 (Jnk-1) (Caraglia et al., 1995, 1999). Moreover, we have also demonstrated that the same cells are strictly dependent from the Ras → Erk-mediated survival signaling that protects them from apoptotic stimuli (Caraglia et al., 2003, 2004, 2005). On the basis of these considerations, in the present study we have evaluated the non-thermal effects of MW-EMF on the apoptosis of human epidermoid cancer cells, that can be a possible biological target of microwaves generated by mobile phones. Since we have found that MW-EMF induced apoptosis in these cells we have studied the modulation of the expression, activity, and proteasome-dependent degradation of the components of ras → Erk- and Akt-dependent survival signaling induced by MW-EMF. Finally, we have investigated on the role of HSP90/multi-chaperone-dependent multi-chaperone complex in the regulation of expression and activity of anti-apoptotic signaling proteins ras and Raf-1 and of their relative survival signaling induced by MW-EMF.

MATERIALS AND METHODS

Materials

DMEM, BSA, and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ). Rabbit antisera raised against βtubulin, Erk-1 K-23 and Erk-2 MAb C-14 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt Mab and the relative activity evaluation kit was purchased by (Cell Signaling Technology, Beverly, MA). Anti-pan-Ras MAb clone 10 and U0126 were purchased from Calbiochem (Darmstadt, Germany). Anti-HSP-70 C92F3A-5 and anti-HSP-90 AC88 monoclonal antibodies were from StressGen Biotech. Co. (Victoria, BC, Canada), and anti-HSP-27 G3.1 MAb from Affinity Bioreagents (Neshanic Station, NJ). Anti-JNK-1 C-17 and anti-$MAPK^{p38}$ C-20 rabbit antisera were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

The human oropharyngeal epidermoid carcinoma KB cancer cell line, obtained from the American Type Tissue Culture Collection (Rockville, MD) was grown in DMEM supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Exposure system

The exposure system was "waveguide" featured: in a cavity box-shaped of ($45 \times 11 \times 5.5$) cm, fed by a microwave generator whose signal is properly amplified, was generated a TE field ("Transverse Electric" that is an electromagnetic wave whose electric field component is orthogonal to the travelling direction); by means of a bi-directional coupler and the power meters linked to it, it is possible to monitor accurately the power developed into the cavity. Inside it, along the principal axis, a flask was positioned, containing KB cells. The waveguide was thermostated at 37°C temperature, kept constant during the whole exposures of 1, 2, and 3 h. The average absorbed power per mass unit (SAR, "Specific Absorption Ratio") was 3.6 ± 0.2 mW/g in each experiment, and the working frequency 1.95 GHz.

Western blot analysis

KB cells were exposed or not for 48 h to MW-EMFs at 37°C. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped, and centrifuged for 30 min at 4°C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate,

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0.1 NaCl, 1mM EDTA, pH 7.5, 10 mM Na_2HPO_4, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, and 0.025 units/ml aprotinin). Equal amounts of cell proteins were separated by SDS–PAGE. The proteins on the gels were electrotransferred to nitrocellulose and reacted with the different MAbs.

Study of the ubiquitination of signaling proteins

KB were seeded to MW-EMFs and/or 10 μM lactacystin for the indicated times. At the time of the assay cells were washed three times with PBS and cell proteins were extracted as described above. For the determination of ras, raf-1 and Erk ubiquitination the supernatants were subjected to immunoprecipitation with anti-ras, anti raf-1, and anti-Erk antibodies. The different proteins were precipitated from 300 μg of cell lysates using 5 μg of MAb for 12 h at 4°C and 50 μl of Protein A Sepharose (Sigma, Milan, Italy) 1:1 suspension for 12 h at 4°C. Immunoprecipitated samples were washed four times with lysis buffer supplemented with 0.1% SDS, boiled in 20 μl Laemmli Buffer for 5 min and electrophorased by 10% SDS–PAGE. Proteins were then electroblotted and probed with the anti-ubiquitin rabbit antiserum (diluted 1:500) FL-76 (Santa Cruz Biotech., CA). The specific bands for ubiquitin were detected with goat anti-rabbit (Santa Cruz, CA) conjugated with peroxidase and subsequent ECL reaction (Amersham, Milan, Italy).

Affinity precipitation of Ras

KB cells were exposed to MW-EMFs as described above. The cells were lysed in the Mg^{2+} buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol. Then, 10 μl ras binding domain (RBD) conjugated to agarose was added to 1 mg of cell lysate and the mixture was incubated at 4°C for 1 h. The agarose beads were collected by microcentrifugation at 14,000*g* for 5 sec and washed three times with Mg^{+2} buffer. The agarose beads were boiled for 5 min in 2× Laemmli sample buffer and collected by a microcentrifuge pulse. The supernatants were run on 12% SDS–PAGE, then the proteins were electrotransferred on a nitrocellulose film. The nitrocellulose was incubated overnight with 1μg/ml of anti-Ras Mab, clone RAS10, and with a secondary Mab, a goat α-mouse HRP conjugated IgG, for 1.5 h. The film was washed with PBS/0.05% Tween 20 and detected by ECL, chemiluminescence's technique, (Amersham).

Internucleosomal DNA fragmentation (ladder)

For all apoptosis evaluation experiments (gel ladder and FACS analysis) both attached and suspended cells were collected prior the processing. DNA fragmentation was measured after extraction of low molecular weight DNA. Briefly, 10×10^6 cells were resuspended in 900 μl, 1× Tris-EDTA buffer and lysed with 25 μl, 20% SDS. DNA was precipitated in ethanol for 6 h in the presence of 5M NaCl. The high molecular weight fraction was sedimented by high-speed centrifugation, and the fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in water, DNA was electrophoresed using 1.5% agarose gel and visualized by ultraviolet light following ethidium bromide staining.

Flow cytometric analysis of apoptosis

Apoptotic cell death was analyzed by Annexin-V-FITC staining and by propidium iodide (PI) detection systems. Annexin-V-FITC binds to phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis. Labeling of apoptotic cells was performed using an Annexin-V kit (MedSystems Diagnostics, Vienna, Austria). Briefly, cells were incubated with Annexin-V-FITC in a binding buffer (provided by the manufacturer) for 10 min at room temperature, washed and resuspended in the same buffer as described by the manufacturer. Analysis of apoptotic cells was performed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA). PI analysis of apoptosis was performed using a commercial kit (MedSystems Diagnostics, Vienna, Austria). The cells were washed in PBS, resuspended in 190 μl of prediluted binding buffer (1:4), and incubated for 10 min with 10 μl of the 20 mg/ml PI stock solution, and then the apoptotic cells were analyzed by FACScan flow cytometer. For each sample, 2×10^4 events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

AKT kinase assay

KB cells were exposed to microwaves as described above. At the time of processing 1 ml ice-cold cell lysis buffer (20 mM TRIS, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptine, and 1 mM PMSF) was added to cells that were incubated on ice for 10 min. The cells were collected and transferred to microcentrifuge tubes and centrifuged at 1,200*g* for 10 min at 4°C. The supernatants were collected and precipitated with 20 μl of Igb1 anti-Akt monoclonal antibody immobilized with agarose beads (Cell Signaling Technology) by o/n incubation with gentle rocking at 4°C. The resulting immunoprecipitates were then incubated for 30 min at 30°C with 1μg GSK-3 fusion protein (Cell Signaling Technology) in the presence of 200 μM ATP and kinase buffer (25 mM TRIS, pH 7.5, 5 mM β glycerophosphate, 2 mM dithiotreitol, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$). The reaction was terminated with the addition of 20 μl, 3× SDS sample buffer. The supernatants were boiled for 5 min and electrophorased by 12% SDS–PAGE and the protein electro-transferred on a nitrocellulose film. Phosphorylation of GSK-3 was detected using as probe an anti-Phospho-GSK-3α/β(Ser21/9) rabbit polyclonal antibody (diluted 1:1,000) and then with a secondary anti-rabbit HRP-conjugated monoclonal antibody (diluted 1:2,000). The film was washed with TBS 1× 0.05% Tween 20 buffer and the specific reactivity was detected by chemiluminescence technique (Amersham, Milan, Italy).

Fluorescence microscopy

After washing in PBS, cells were treated with in situ detection kit, according to manufacturers (SantaCruz Biotechnology, CA). In details, cells were incubated with PI and a FITC-conjugated antibody raised against annexin V for at 37°C at the dark. Then, cells were observed under fluorescent microscope using a dual filter set for FITC and rhodamine. The images were acquired with a dedicated sotware.

Transfection by electroporation

Cells were detached from confluent 100 mm-dishes. 100×10^6 cells were incubated in appropriate electroporation vials with 800 μl of electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose) and 15 μg of the the previously described RASN17 DNA or of p2HG/hHsp90β DNA in 20 mM HEPES (Donzé and Picard, 1999; Caraglia et al., 2003). Then cells were electroporated at 250 volts and at 975 μF for 6 sec. The cells were incubated at 37°C with or without 1,000 IU/ml IFNα for 24 h. After the incubation the cells were processed for FACS analysis as described above.

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed by analysis of variance (ANOVA) with Neumann–Keul's multiple comparison test or Kolmogorov–Smirnov where appropriate.

RESULTS

MW-EMF induces apoptosis of human epidermoid KB cancer cells

It has been demonstrated that MW-EMFs can induce apoptosis in some cancer cells and we have reported that MW-EMFs can affect the three dimensional structure of eukaryotic proteins (Maeda et al., 2004; Mancinelli et al., 2005 in press). On the basis of these considerations we have evaluated if MW-EMFs can also induce apoptotic effects on human epidermoid cancer KB cells derived from a squamous head and neck cancer. We have

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indeed found that MW-EMFs can induce a time-dependent increase of the number of apoptotic KB cells as demonstrated with the FACS analysis after labeling with FITC Annexin V. In fact, after 1 h of exposure of KB cells to MW-EMFs about 20% of cells were apoptotic (Fig. 1B). After 2 and 3 h of exposure to MW-EMFs apoptosis was found in about 32%–45% of cell population, respectively (Fig. 1C,D respectively). Similarly, the exposure of to MW-EMFs induced the typical pattern of DNA fragmentation that occurs during apoptotic onset as evaluated with the analysis of internucleosomal DNA fragmentation (Fig. 1E). The latter effect was again time-dependent. We have also analyzed the apoptotic effects of MW-EMF on KB cells at fluorescence microscopy with double PI and FITC-annexin V labeling. We have again found a time-dependent increase of the green fluorescent cells in MW-EMF-treated cells thus suggesting the occurrence of apoptosis (Fig. 1A–D). These data demonstrate that MW-EMF can induce apoptosis in human epidermoid cancer cells.

Effects of MW-EMF on the components of the survival signaling

We have previously demonstrated that human epidermoid KB cells are strictly dependent from the Ras → Erk-mediated survival signaling pathway that protects KB cells from apoptotic stimuli (Caraglia et al., 2003, 2005). On the basis of these considerations we have evaluated the effects of MW-EMF on the expression and activity of the components of the ras → Erk-dependent pathway. We have found that MW-EMF induced a time-dependent decrease of the expression and activity of ras as evaluated with Western blotting analysis and with precipitation for affinity with the minimal binding domain of raf-1, respectively (Fig. 2A,B respectively). In fact, an about 3-fold reduction of both ras expression and activity were recorded after 3 h exposure of KB cells to MW-EMF (Fig. 2A,B respectively). At the same time, an about 2-fold decrease of Raf-1 expression was also recorded (Fig. 2C). On the other hand, the exposure of KB cells to MW-EMF did not induce any change in the expression of Erk-1 and Erk-2 as determined with Western blotting (Fig. 2D); however, an about 2-fold decrease of the activity of Erk-1 and Erk-2 was again found at the same experimental conditions as evaluated with Western blotting of the phosphorylated isoforms of Erk-1/2 (Fig. 2E). The latter effects suggest that the decrease of the activity of the anti-apoptotic key enzymes Erk-1/2 was determined by the decrease of expression and activity of the upstream enzymes ras and Raf-1. Thereafter, we have evaluated the effects of MW-EMF on another important survival pathway regulated by EGF and ras, the Akt/PKB signaling. In details, we have studied both Akt expression and activity with Western blotting and in gel kinase assay, respectively. A significant and time-dependent reduction of the expression of Akt was also detected in MW-EMF treated KB cells (about 2-fold decrease after 3 h exposure to MW-EMF) (Fig. 2F). However, the activity of Akt was unchanged in MW-EMF-treated cells (Fig. 2G). The latter effect was paralleled by a 2-fold increase of the expression of PI3K, the upstream activator of Akt, thus suggesting an Akt hyperactivation by PI3K (Fig. 2H). Notably, MW-EMF did not cause any

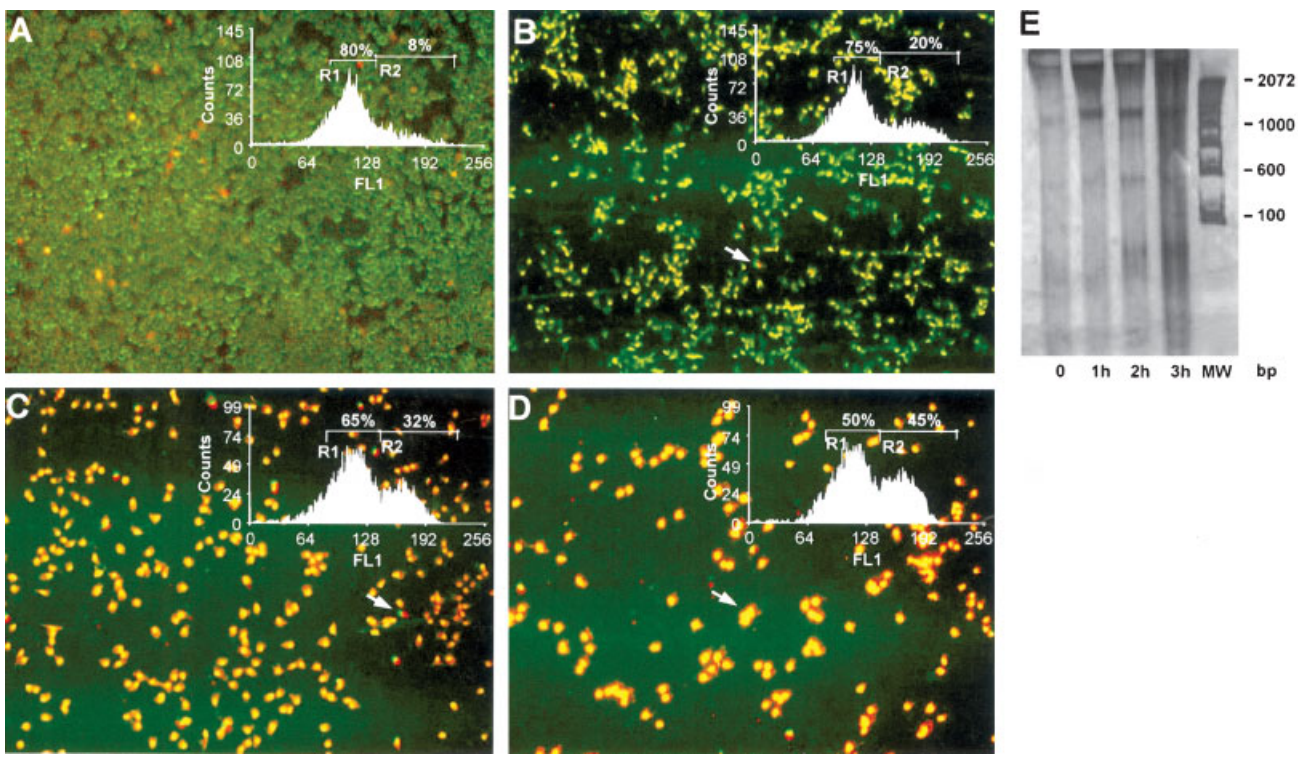

Fig. 1. MW-EMF induces apoptosis in human epidermoid KB cancer cells. KB cells were seeded and exposed to MW-EMF for 1, 2, and 3 h as described in Materials and Methods. Subsequently, we have evaluated the apoptotic effects of MW-EMF on KB cells at fluorescence microscopy after labeling with PI and anti-annexin V antibody (photos) and at FACS analysis after anti-annexin V antibody labeling (insets). (**A**) Control cells; (**B**) cells exposed to MW-EMF for 1 h; (**C**) cells exposed to MW-EMF for 2 h; (**D**) cells exposed to MW-EMF for 3 h. The experiments were performed at least three times and the results were always similar. Red and green fluorescent cells were apoptotic. The bars in the insets show the percentage of apoptotic cells. Arrows show apoptotic cells. (**E**) The internucleosomic DNA fragmentation was assessed as described in Materials and Methods. The exposure of KB cells to MW-EMF induced apoptosis in a time-dependent fashion. The experiments were performed at least three times and the results were always similar. 0, untreated; 1 h, cells exposed to MW-EMF for 1 h; 2 h, cells exposed to MW-EMF for 2 h; 3 h, cells exposed to MW-EMF for 3 h. MW, molecular weights.

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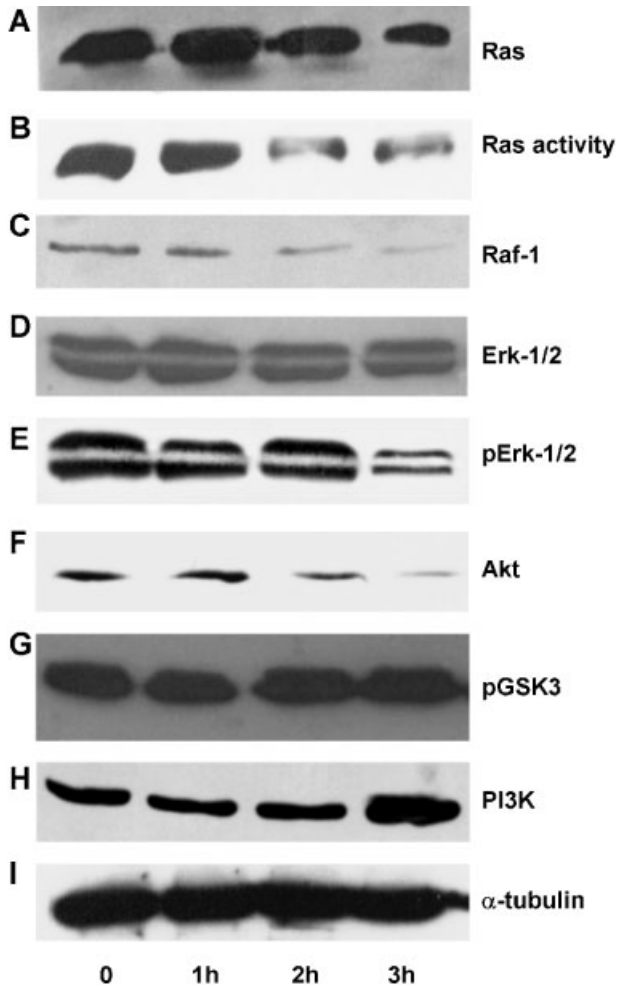

Fig. 2. Effects of MW-EMF on the components of the survival signaling. KB cells have been exposed to MW-EMF for different times and thereafter were processed for the determination of the expression and activity of different components of survival signaling. (**A**) Western blot assay for the expression of the total ras protein. (**B**) Affinity precipitation of ras performed with the minimal binding domain of raf-1 conjugated with agarose for the evaluation of ras activity as described in "Materials and Methods." Expression of Raf-1 (**C**) and Erk-1 and 2 (**D**) and phosphorylation (**E**) of erk-1 and 2 evaluated after blotting with an anti-Raf-1, anti-MAPK and an anti-pMAPK specific Mab, respectively, as described in "Materials and Methods." In the same experimental conditions the expression (**F**) and activity (**G**) of Akt was also analyzed with a Western blotting and an in-gel kinase assay, respectively, as described in "Materials and Methods." (**H**) The expression of PI3K was evaluated with Western blotting assay using an appropriate antibody as described in "Materials and Methods." (**I**) Expression of the house-keeping protein α-tubulin, used as loading control. The experiments were performed at least three different times and the results were always similar. 0, untreated; 1 h, cells exposed to MW-EMF for 1 h; 2 h, cells exposed to MW-EMF for 2 h; 3 h, cells exposed to MW-EMF for 3 h.

change of the expression of the house-keeping protein α-tubulin (Fig. 2I). These data suggest that the apoptosis induced by MW-EMF could be mediated by the decrease of the activity of Erk-1/2 caused by the decrease of the expression and activity of ras and Raf-1.

MW-EMFs modulate the ubiquitin-dependent degradation of ras and raf-1 by proteasome complex

We have found that MW-EMF cause a perturbation of the normal folding of eukaryotic proteins (Mancinelli et al., 2005 in press). Moreover, misfolded and/or unfolded proteins are normally degraded by the cells via an ubiquitin-dependent pathway (Hochstrasser, 1995; Hershko et al., 2000). On the basis of these considerations, we have evaluated if the reduction of the expression of some components of the survival signaling induced by MW-EMF could be due to an increase of their degradation via a proteasome-dependent pathway. We have found that the ubiquitination of both Ras and Raf-1 was 2-fold increased in KB cells exposed to MW-EMF for 3 h as evaluated with Western blotting for ubiquitin after ras and Raf-1 immunoprecipitation, respectively (Fig. 3A,B respectively). This effect was again paralleled by a 2-fold decrease of the expression of the two proteins (Fig. 3D,E). Subsequently, KB cells have been exposed to MW-EMF and/or the specific proteasome inhibitor lactacystin in order to evaluate the effects on ras and Raf-1 ubiquitination and expression. The addition of 10 μM lactacystin for 12 h, inhibiting proteasome-dependent degradation of ubiquitinated ras and Raf-1, caused an accumulation of the ubiquitinated forms of the enzymes (Fig. 4A,B respectively) and a consequent increase of their expression (Fig. 4D,E respectively). The synchronous treatment of the cells with lactacystin and MW-EMF potentiated the accumulation of the ubiquitinated isoforms of ras and Raf-1 induced by lactacystin (Fig. 4A,B respectively). In these experimental conditions a restoration of ras and Raf-1 expression was recorded (Fig. 4D,E respectively). At the same time, we have evaluated the effects of lactacystin and MW-EMF on the ubiquitination and expression of Erk-1/2. We have found that MW-EMF did not modulate either the expression or the ubiquitination of Erk-1/2 (Fig. 3C,F respectively). However, lactacystin induced an increase of the ubiquitination and expression of Erk-1/2 that was not affected by the concomitant exposure to MW-EMF (Fig. 3C,F respectively).

These data suggest that ras, Raf-1, and Erk-1/2 were ubiquitinated and degraded by a proteasome pathway in KB cells and that the exposure to MW-EMF increased poly-ubiquitination and, consequently, degradation of ras and Raf-1, but not of Erk-1/2.

MW-EMFs inactivate the HSP90/multi-chaperone complex

We have found that MW-EMF activated the ubiquitination and proteasome-dependent degradation of survival signal molecules ras and Raf-1. It has been reported that microwaves derived from mobile phone activate HSP27/p38MAPK stress pathway and induce HSP70 (Leszczynski et al., 2002; Shallom et al., 2002). Moreover, HSP90/multi-chaperone complex prevents proteasome-mediated degradation of several signaling molecules including Raf-1 and Akt (Blagosklonny, 2002; Pratt and Toft, 2003). On the bases of these considerations, we have studied the effects of MW-EMF on the expression of HSP90, 27 and 70 by Western blot assay. We have found that HSP27 was up-regulated by the exposure of KB cells to MW-EMF with a maximal 3.5-fold increase at 1 h and at more prolonged exposure no further increase of the protein expression was found (Fig. 4A). HSP70 was also up-regulated by the exposure of KB cells to MW-EMF with a maximal increase at 2 h (Fig. 4B). On the other hand, MW-EMF induced a time-dependent decrease of the HSP90 expression that was stronger (5-fold) at 3 h (Fig. 4C). Moreover, we have evaluated the effects of MW-EMF on JNK-1 and p38 kinase activity, key enzymes of a stress-induced pathway, in KB cells with Western blot assay using antibodies raised against the phosphorylated isoforms

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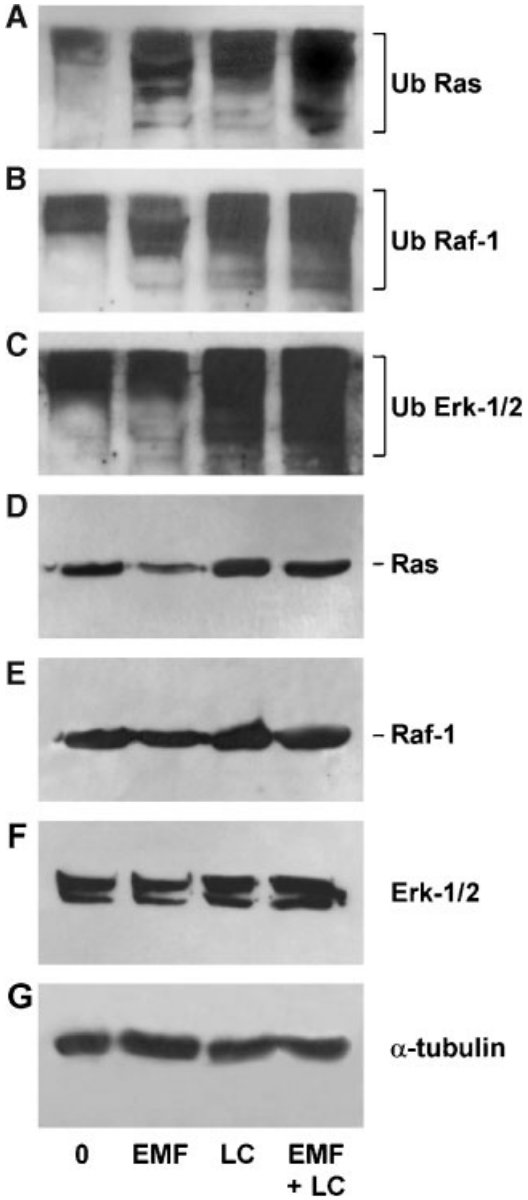

Fig. 3. MW-EMFs modulate the ubiquitin-dependent degradation of ras and raf-1 by proteasome complex. KB cells have been cultured for 12 h in the presence or absence of 10 μM lactacystin (specific proteasome inhibitor) and exposed to MW-EMF for the selected times. Then cellular proteins were extracted and immunoprecipitated with an anti-ras MAb (**A**) or anti-Raf-1 (**B**) or anti-Erk-1/2 MAb (**C**) as described in "Materials and Methods." The immunoprecipitated was subsequently run in SDS–PAGE and immunoblotted for ubiquitin. The expression of total ras (**D**), Raf-1 (**E**), Erk-1/2 (**F**) and α-tubulin (**G**) was also determined with Western blotting assay using specific antibodies as previously described in "Materials and Methods." The experiments were performed at least three different times and the results were always similar. 0, untreated; EMF, cells exposed to MW-EMF for 3 h; LC, cells exposed to 10 μM lactacystin for 12 h; EMF + LC, cells exposed to 10 μM lactacystin for 12 h and MW-EMF for 3 h.

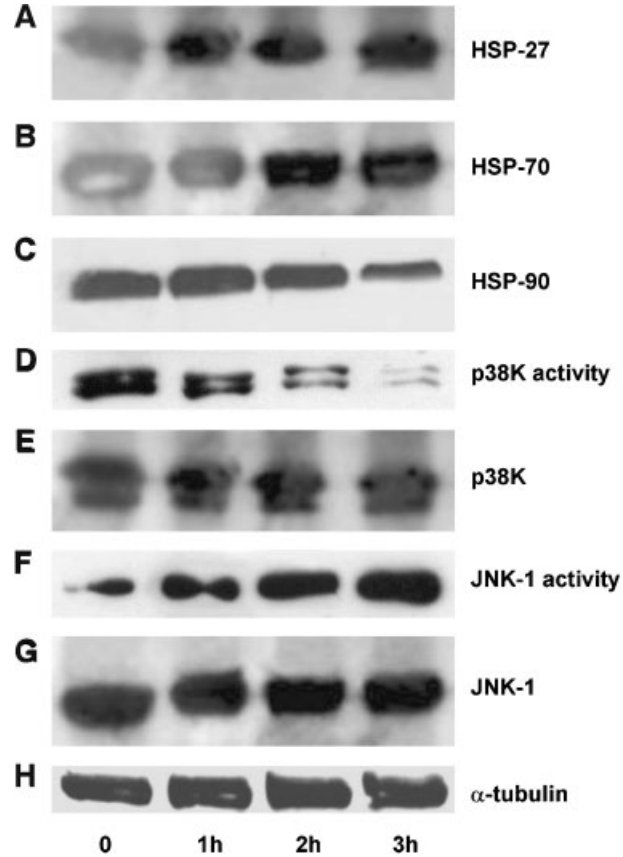

Fig. 4. MW-EMFs inactivate the multi-chaperone complex. KB cells have been exposed to MW-EMF for different times and thereafter were processed for the determination of the expression and activity of different components of stress-activated pathways. Fifty microgram of cell proteins/lane have been assessed by Western Blot analysis after electrotransfer to nitrocellulose filter of whole cell lysates, which have been separated by PAGE and hybridized with anti-HSP-27 G3.1 (**A**) or anti-HSP-70 C92F3A-5 (**B**) or anti-HSP-90 AC88 MAbs (**C**). Similarly the elctrotransfered proteins were hybridized with anti-phospho 38 kinase (**D**) and anti-pJNK1 (**F**) and with anti-$MAPK^{p38}$ (**E**), anti-JNK-1 C-17 C-20 rabbit antisera (**G**) and anti-tubulin-α Mab. Specific MAb binding has been detected by ECL following HRP-linked anti-mouse antibody blotting. The experiments were performed at least three different times and the results were always similar. 0, untreated; 1 h, cells exposed to MW-EMF for 1 h; 2 h, cells exposed to MW-EMF for 2 h; 3 h, cells exposed to MW-EMF for 3 h.

of the two enzymes. We have found that MW-EMF induced a time-dependent decrease of the activity of p38 kinase and this effect was maximal (4-fold) after 3 h of exposure (Fig. 4D). On the other hand, MW-EMF induced an about 2.5-fold increase of the activity of JNK-1 and this effect was higher after 1 h of exposure and no further increase was detected at more prolonged time (Fig. 4F). At these experimental conditions, MW-EMF did not induce any change in the expression of total p38 kinase and JNK-1 as evaluated with Western blotting using antibodies raised against both phosphorylated and unphosphorylated isoforms (Fig. 4E,G respectively). These results suggest that MW-EMF induced differential pattern of stress kinase activation in KB cells and it strongly suppressed the expression of HSP90 and likely inactivated the HSP90/multi-chaperone complex.

HSP90 restoration antagonizes apoptosis and decreased Erk-1/2 activity induced by MW-EMFs in human epidermoid cancer cells

In order to demonstrate that the inactivation of the HSP90/multichaperon complex by MW-EMF is involved in the degradation of the components of the ras → Erk signaling and in the induction of apoptosis, KB cells were transfected with a plasmid encoding for HSP90 as described above. After transfection, the cells were treated or not for 24 h with 10 μM of the specific inhibitor of MEK-1 (the upstream activator of Erk-1/2) U0126. The cells were exposed to MW-EMF after 24 h from the

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transfection since at this time the overexpression of HSP90 was maximal (data not shown). Control cells were transfected with an irrelevant DNA. After 3 h of exposure to MW-EMF the cells were labeled with FITC annexin V and analyzed at FACScan. The exposure of parental KB cells to MW-EMF for 3 h induced again about 45% apoptosis while the transfection of HSP90, as expected, had no effect on the occurrence of apoptosis (6% vs. 7% in untreated parental cells) (Fig. 5B,C respectively). The transfection and the consequent overexpression of HSP90 almost completely antagonized the apoptosis induced by MW-EMF. In fact, only 10% of cell population was apoptotic in HSP90-transfected KB cells exposed to MW-EMF (Fig. 5D). The treatment of KB cells with U0126 for 24 h caused apoptosis in about 36% of parental cell population unexposed to MW-EMF and in about 65% of parental cells exposed to MW-EMF (Fig. 5E,F respectively). Moreover, U0126 completely antagonized the counteracting effect of HSP90 transfection on apoptosis induced by MW-EMF. In fact, 42% of HSP90-transfected cell population exposed to MW-EMF for 3 h was again apoptotic (Fig. 5G). Notably, U0126 induced about 30% apoptosis in unexposed HSP90-transfected KB cells (data not shown). In these experimental conditions MW-EMF was again able to reduce the expression of HSP90, ras and Raf-1 and the

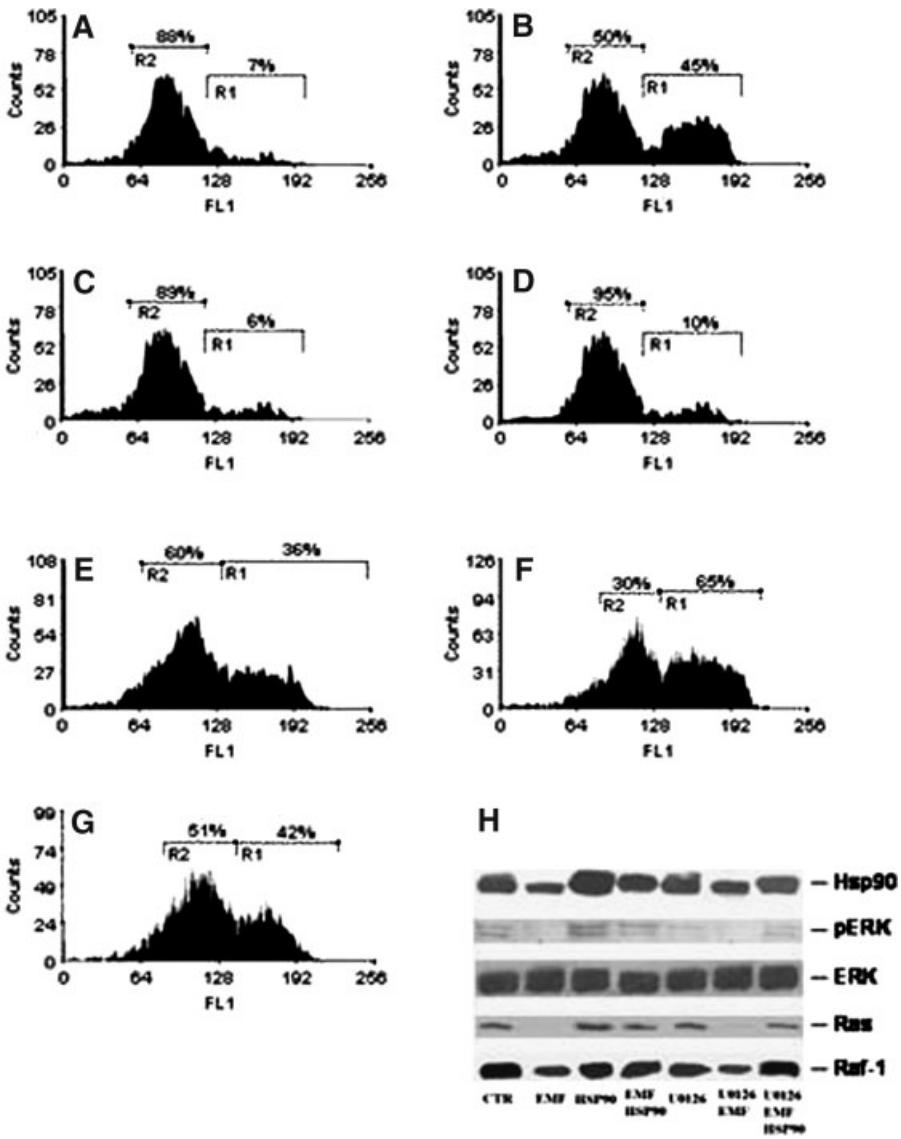

Fig. 5. HSP90 restoration antagonizes apoptosis and decreased Erk-1/2 activity induced by MW-EMFs in human epidermoid cancer cells. (**A**–**G**) FACS analysis of parental or HSP90-transfected KB cells exposed to MW-EMF for 3 h and/or 10 μM U0126 for 12 h. Cells have been collected, labeled with FITC annexin V and analyzed as described in "Materials and Methods." (A) Untreated parental cells; (B) Untreated parental cells exposed to MW-EMF for 3 h; (C) Untreated HSP90-transfected cells; (D) HSP90-transfected cells exposed to MW-EMF for 3 h; (E) parental cells exposed to 10 μM U0126 for 12 h; (F) parental cells exposed to 10 μM U0126 for 12 h and to MW-EMF for 3 h (G) HSP90-transfected cells exposed to 10 μM U0126 for 12 h and to MW-EMF for 3 h. The experiments were performed at least three times and the results were always similar. The bars show the percentage of apoptotic cells. (H) Parental or HSP90-transfected KB cells have been exposed to MW-EMF or U0126 as described above and thereafter were processed for the determination of the expression of HSP90, pErk-1/2, Erk-1/2, ras and Raf-1 with Western blotting assay as described in "Materials and Methods." The experiments were performed at least three different times and the results were always similar. CTR, Untreated parental cells; EMF, Untreated parental cells exposed to MW-EMF for 3 h; HSP90, Untreated HSP90-transfected cells; EMF HSP90, HSP90-transfected cells exposed to MW-EMF for 3 h; U0126, parental cells exposed to 10 μM U0126 for 12 h; U0126 + EMF, parental cells exposed to 10 μM U0126 for 12 h and to MW-EMF for 3 h; U0126 + EMF HSP90, HSP90-transfected cells exposed to 10 μM U0126 for 12 h and to MW-EMF for 3 h.

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activity of Erk1/2 (Fig. 5H, EMF lane). On the other hand, HSP90 was over-expressed (3-fold) in untreated and restored (resembling the expression in untreated cells) in MW-EMF-treated HSP90-transfected cells (Fig. 5H, HSP90 and EMF HSP90, respectively). The MW-EMF-treated HSP90-transfected cells showed ras and Raf-1 intracellular levels and Erk-1/2 activity similar to that one of control parental cells as demonstrated by the Western blotting (Fig. 5H, EMF HSP90). Conversely, U0126 treatment had no effect on HSP90, ras and Raf-1 expression both in parental and transfected cells (Fig. 5H, U0126), but it prevented the restoration of Erk activity in HSP90-transfected cells exposed to MW-EMF (Fig. 5H, U0126 + EMF HSP90). These results suggest that MW-EMF induced apoptosis through the inactivation of the HSP90/multi-chaperone complex and the consequent degradation of Ras and Raf-1. The latter effect can lead to the inactivation of the anti-apoptotic ras → Erk signal transduction pathway.

DISCUSSION

Mobile phone use has dramatically increased in the last years (Repacholi, 2001) and as a consequence there is an increasing public interest about health hazard due to radiofrequency fields exposure (Hyland, 2000; Laurence et al., 2000; Adair, 2002). On the other hand, the use of microwaves is emerging as an additional non-invasive approach for the therapy of human neoplasms, also taking advantage from the generation of shock responses and apoptosis in human cancer cells (Maeda et al., 2004).

Although structural and functional properties of native proteins could not be affected by the presence of MW-EMF (Adair, 2002) we have recently demonstrated that microwaves generated by an electromagnetic field can affect the three dimensional structure of eukaryotic proteins suggesting also possible biological effects in living cells (Mancinelli et al., 2005 in press). In fact, misfolded and/or unfolded proteins are degraded by the eukaryotic apparatus in order to avoid non-productive interactions that would result in aggregation and fibrin formation that could cause cellular damage. Protein degradation can be driven by the proteasome-dependent pathway following the covalent addition to the proteins of several small molecules of 14 kDa, called ubiquitin (Hochstrasser, 1995; Hershko et al., 2000). This event leads to the subsequent delivery of the protein to a macromolecular complex called proteasome that determines the final proteolysis and degradation of the protein (Hershko et al., 2000). On the other hand, mechanisms that prevent the unfolding and the consequent degradation of intracellular proteins exist. One of these mechanisms is regulated by HSPs that could be involved as molecular chaperones to rescue damaged proteins and subtracting them from the proteasome-dependent degrading pathway (De Pomerai et al., 2000; French et al., 2000; Hyland, 2000; Leszczynski et al., 2002). In fact, Hsp90 is one of the most abundant cellular chaperone proteins. It functions in a multicomponent complex of chaperone proteins that may include p60/Hop, p50Cdc37, Hsp40/HDJ2, p23, BAG-1, Hsp70, and one variety of a immunophilins (Goetz et al., 2003). It forms the basis of a super-chaperone machine that promotes the proper folding of client proteins so that they can respond to a stimulus or bind ligand. However, the machine is in constant flux and cycles between two Hsp90 conformations, determined by ATP or ADP binding, which in turn specify which set of cochaperones associate with the chaperone complex (Isaacs et al., 2003). Cycling of this machine is driven by ATP hydrolysis. Although Hsp90 is a weak ATPase, its activity is regulated by cochaperones and dramatically enhanced by client protein binding. The binding of HSP70 to the multichaperon complex favors the ATP hydrolysis and makes the client protein susceptible to ubiquitination and delivery to the proteasome (where it is degraded) (Isaacs et al., 2003). HSP 90 has been specifically involved in the maintainance of the correct conformation of several intracellular proteins (named HSP90 clients) and much of them are kinases involved in the control of cell proliferation and survival, such as Raf-1 and Akt (Mayer and Bukau, 1999).

The Hsp90-dependent kinases are likely to be structurally unstable—this may be hard to avoid in proteins that have to undergo structural transitions in their roles as molecular switches. They are multidomain proteins, of which the carboxy-terminal catalytic domain is conserved in sequence and structure within the different kinase classes. They undergo signal-induced conformational changes, which in the case of some of the kinases are known to as a consequence of low Hsp90 levels. This conformational flexibility may involve chaperone-dependent folding transitions and conformational states. Furthermore, some kinases need hydrophobic cofactors, and in these cases Hsp90 may hold the kinase in the proper conformation for receiving them (Mayer and Bukau, 1999). These aspects of multi-chaperone function has encouraged its targeting with specific drugs that bind the multi-chaperone complex at the nucleotide binding region such as geldanamycin and its clinical grade derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG). The 17-AAG-bound conformation Hsp90, resembles the chaperone's ADP-bound conformation, GA binding promotes stable assembly of the super-chaperone machine that favors client protein degradation (Schneider et al., 1996). Phase I/II clinical trials with 17-AAG are ongoing on patients affected by several neoplasms and in one of these the analysis of pharmacodynamic markers in peripheral blood mononuclear cells (PBMCs) at 450 mg/m^2/week, showed a reduction in the expression of Raf-1 between 24 and 48 h and Hsp70 induction at 24–48 h (Banerji et al., 2002; Goetz et al., 2003). Interestingly, all the available phase I studies accomplished that 17-AAG can be administered with tolerable toxicity and reduction of expression of HSP90 clients (Bagatell and Whitesell, 2004). This evidence suggests the hypothesis that multi-chaperone is in a hyperactivated state only in cancer cells making the action of 17-AAG specific for tumor cells.

On the basis of the sensitivity of several kinases to unfolding conditions, it can be also suggested that this class of protein kinases could be more susceptible to environmental stresses, including MW-EMF, that could affect their proper folding and determine their degradation, in the absence of appropriate levels of HSP90. In the present investigation we have indeed found that MW-EMF induced apoptosis paralleled by a decreased expression of classical HSP90 clients such as Raf-1 and Akt in human epidermoid cancer KB cells. While the activity of the downstream Raf-1 targets Erk-1 and Erk-2 was decreased, Akt activity was not reduced likely as a consequence of the increased expression of the upstream activator PI3K. Moreover, we have also found a reduction of the expression and, consequently, of the activity of ras that until today, at our knowledge, has not been still described as HSP90-chaperone client. On the basis of these results, we have hypothesized that

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the decreased expression of ras and Raf-1 could be due by an increase of their ubiquitination and proteasome dependent degradation. We have found that MW-EMF increased the ubiquitination of the two enzymes and the proteasome inhibition induced by lactacystin caused an accumulation of the ubiquitinated isoforms of the two enzymes and counteracted the effects of MW-EMF on ras and Raf-1 expression. These effects were paralleled by a differential pattern of stress pathway activation. In fact, MW-EMF determined an increase of JNK-1 activity and HSP70 and HSP27 expression and a decrease of p38 kinase activity and HSP90 expression. On the basis of these results, we hypothesized that the reduced expression and activity of ras and Raf-1, and of the pathway activated by them, could be due to the decreased expression of the HSP90 and to the consequent targeting of the two proteins to the proteasome-dependent degradative machinery. In order to support this hypothesis, we have transfected KB cells with a plasmid encoding for HSP90 and able to over-express the protein. We have indeed found that HSP90 over-expression protected KB cells from apoptosis induced by MW-EMF and the treatment of the cells with a specific Mek-1, and therefore Erk, inhibitor U0126 was able to antagonize the anti-apoptotic effect induced by HSP90 hyper-expression. The protective effect induced by HSP90 was paralleled by the rescue of ras and Raf-1 expression and of Erk-1/2 activity.

In conclusion, these results demonstrate for the first time that MW-EMF induced apoptosis through the inactivation of the ras → Erk survival signaling due to enhanced degradation of ras and Raf-1. The latter effect is due to the decreased expression of HSP90 and the consequent increase of the proteasome dependent degradation of its client proteins. These data could be useful for the understanding of the biological non-thermal and environmental effects of radiofrequency generated by mobile phone. Moreover, taking into account the emergence of therapeutic anti-cancer approaches based on the selective inhibition of HSP90/multi-chaperone complex by 17-AAG (Goetz et al., 2003; Maeda et al., 2004) the use of MW-EMF could represent an additional strategy in order to potentiate the activity of this agent.

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