EUROPEAN JOURNAL OF CANCER 47 (2011) 2042–2051

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Apigenin affects leptin/leptin receptor pathway and induces cell apoptosis in lung adenocarcinoma cell line

Andreina Bruno [a,*], *Liboria Siena* [a], *Stefania Gerbino* [a], *Maria Ferraro* [a], *Pascal Chanez* [b], *Marco Giammanco* [c], *Mark Gjomarkaj* [a], *Elisabetta Pace* [a]

[a] *Institute of Biomedicine and Molecular Immunology, National Research Council, Palermo, Italy*
[b] *Département des Maladies Respiratoires, U600 INSERM and AP-HM, Université de la Méditerranée, Marseille, France*
[c] *Department of Medicine, Pneumology, Physiology and Human Nutrition, University of Palermo, Palermo, Italy*

ARTICLE INFO

Article history:
Received 16 February 2011
Accepted 30 March 2011
Available online 5 May 2011

Keywords:
Apigenin
Apoptosis
Leptin
Lung cancer
Pleural fluids
Proliferation
ROS

ABSTRACT

Background: Apigenin, a common edible plant flavonoid, is a well characterised antioxidant. The adipokine leptin exerts proliferative and anti-apoptotic activities in a variety of cell types. In cancer cells, apigenin may induce a pro-apoptotic pathway whereas leptin has an anti-apoptotic role. The purpose of the study is to investigate the role of apigenin and of leptin/leptin receptor pathway on proliferation and on apoptosis in lung adenocarcinoma.

Methods: Immunocytochemistry, flow cytometry and RT-q-RT PCR, were used to investigate the expression and modulation of leptin receptors on the lung adenocarcinoma cell line A549 in presence or absence of apigenin and of leptin, alone or combined. Clonogenic test to evaluate cell proliferation was assessed. Exogenous leptin binding to its receptors by flow cytometry, reactive oxygen species (ROS) by dichlorofluorescein diacetate analysis, cell death by ethidium bromide and apoptosis by annexin V analysis were assessed. Apoptosis was assessed also in presence of lung adenocarcinoma pleural fluids (PF) ($n = 6$).

Results: A549 express leptin/leptin receptor pathway and its expression is upregulated by apigenin. Apigenin alone or combined with leptin significantly decreases cell proliferation and significantly increases the spontaneous release of ROS, with augmented cell death and apoptosis, this latter also in the presence of lung adenocarcinoma PF. Leptin alone significantly increases cell proliferation and significantly decreases cell death.

Conclusions: These results strongly suggest the potential utility of the flavonoid apigenin in the complementary therapeutic approach of patients with lung adenocarcinoma.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide and non-small cell lung cancer (NSCLC) accounts for >80% of all lung cancers.[1]

Epidemiological studies suggest a protective effect of high dietary intake of flavonoids with fruits and vegetables against cardiovascular diseases and cancer, including the lung cancer.[2,3] It is supposed that fruits and vegetables contain and release compounds that have protective effects, independent

* *Corresponding author.* Address: Institute of Biomedicine and Molecular Immunology, National Research Council, Via Ugo La Malfa, 153-90146 Palermo, Italy. Tel.: +39 091 680 9180; fax: +39 091 680 9122.
E-mail addresses: andreina@neomedia.it (A. Bruno), siena@ibim.cnr.it (L. Siena), stefania1201@libero.it (S. Gerbino), maryferraro@libero.it (M. Ferraro), pascal.chanez@univmed.fr (P. Chanez), giammanco@unipa.it (M. Giammanco), gjomarkaj@ibim.cnr.it (M. Gjomarkaj), pace@ibim.cnr.it (E. Pace).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.03.034

of those of known nutrients and micronutrients. Plant flavonoids are a large family of plant metabolism products. Because of differences in their chemical structure, bioavailability, distribution and metabolism, different flavonoid compounds may have different effects on human health.[4] Especially, apigenin (4′,5,7,-trihydroxyflavone) is a flavonoid that has been shown to promote apoptosis in various human malignant cell lines and to have preventive and therapeutic potential against human malignant tumours. Apigenin induces loss of mitochondrial transmembrane potential, increase of reactive oxygen species (ROS) production, release of mitochondrial cytochrome *c* into the cytosol with subsequent induction of apoptosis in leukaemia cells.[5] The potency of flavonoids for inducing apoptosis may be dependent on different structure–activity relationship. On the other hand leptin, originally described as an adipocyte-derived hormone regulating the food intake and the energy expenditure, is a pleiotropic hormone with a proliferative and an anti-apoptotic role involved in several systems,[6] including the lung cancer.[7,8] Leptin exerts its action via its receptors (Ob-R, long and short isoforms) and functional leptin receptors are found to be expressed on diverse cells derived from different tissues[9] including the lung.[10,11] Studies performed *ex vivo* in endobronchial biopsies from patients affected by chronic obstructive pulmonary disease (COPD)[10] and by bronchial asthma[11] showed that leptin/leptin receptor pathway is increased in the submucosa while it is decreased in airway epithelium of these patients. Leptin/leptin receptor pathway in primary bronchial epithelial cells exerts a role in maintaining epithelial homeostasis[11] and in endothelial cells leads to ROS formation and cell proliferation.[12] Furthermore, in *ob/ob* mice, it has been shown that the leptin receptors are over-expressed in bronchial epithelial cells, pneumocytes, alveolar macrophages and bronchial/vascular smooth muscle cells in presence of cigarette smoke (CS).[13] Additionally, in these mice, exogenous leptin administration completely restored the skewed inflammatory profile, underlying an important role of leptin in modulating innate and adaptive immunity after CS inhalation.[13]

Based on all these evidences, the authors of the present study aimed to investigate *in vitro* the role of apigenin and of the leptin/leptin receptor pathway, in cell proliferation, cell death and apoptosis using the lung adenocarcinoma cell line A549 and the lung adenocarcinoma pleural fluids (PF).

2. Materials and methods

2.1. A549 cultures and treatments

A549 cell line were purchased from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in complete culture medium (RPMI 1640 containing 10% FCS and 200 IU/mL penicillin/100 μg/mL streptomycin) in the presence or absence of apigenin (50 μM; Sigma–Aldrich, Milan, Italy) or of recombinant human leptin (0.5 μM; R&D Systems), alone or combined, for evaluating leptin receptor expression, by immunocytochemistry, flow cytometric analysis and by RT-PCR and for evaluating leptin by flow cytometric analysis. Furthermore, cells were evaluated for cell proliferation, for ROS generation, for cell death and apoptosis. This latter was evaluated also in the presence or absence of lung adenocarcinoma PF obtained from cancer patients. Experiments were performed from 3 to 6 times for 24, 48 and 72 h. For all treatments the best time was 72 h, except for ROS generation assessment (24 h).

2.2. Immunocytochemistry for leptin receptors

Cytospins were prepared from A549 cell line cultured without any stimulation and cells were fixed and permeabilised as previously described.[6] After washing in PBS, slides were incubated with the primary goat-polyclonal antibody Ob-R anti-leptin receptor against the common part of the short and long isoform (M-18) (1:20 dilution, overnight 4 °C, Santa Cruz Biotechnology, Santa Cruz, Calif.). The reaction was revealed by LSAB KIT phosphatase method (DAKO Glostrup Denmark). Non-immune IgG at the same titre as the primary antibody were used as a negative controls. Cytospins were examined under light microscopy with a final magnification of 1000×.

2.3. Flow cytometric analysis for leptin receptors and leptin

A549 cell line were analysed for leptin/leptin receptor expression by FACS analysis, in presence or absence of apigenin and of leptin, alone or combined. For detection of leptin receptors, A549 cells were stained with the anti leptin receptor antibody Ob-R, 1 h, 4 °C. Non-immune IgG at the same titre as the primary antibody was used as a negative control. Cells were then washed in cold PBS and incubated with FITC-conjugated polyclonal rabbit anti-goat Ig (DAKO Glostrup, Denmark) in the dark 30 min at 4 °C. After washing, the cells were analysed by FACScalibur (Becton Dickinson, Mountain View, CA, USA) flow cytometer. For detection of intracellular leptin, the cells were treated with monensin 1 mM 12 before the cell collection. Afterwards, cells were washed in incubation buffer (PBS containing 1% FCS and 0.1% Na azide) and then incubated with PBS containing 4% paraformaldehyde for 20 min. Fixation was followed by two washes in permeabilisation buffer with PBS containing 1% FCS, 0.3% saponin, and 0.1% Na azide and fixed permeabilised cells were stained with the primary rabbit-polyclonal antibody anti leptin (Ob, A-20, Santa Cruz Biotechnology, Santa Cruz, Calif.), 1 h at 4 °C. Non-immune IgG at the same titre as the primary antibody was used as a negative control. Cells were then washed in incubation buffer and incubated with FITC-conjugated polyclonal swine anti-rabbit Ig (DAKO Glostrup Denmark) in the dark 30 min at 4 °C before flow cytometric analysis. Fluorescence-positive cells were quantified. Percentages of positive cells for leptin receptors and geomean of positive cells for leptin were determined from forward (FS) and sideways (SS) scatter patterns, after gating on the cells, excluding debris. Non-specific binding and background fluorescence were quantified by analysing negative control.

2.4. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) of leptin receptors

Total RNA was extracted from A549 cells, cultured in presence or absence of apigenin and of leptin, alone or com-

bined, with TRIzol Reagent (Invitrogen) according to the method of Chomczynski and Sacchi[14] and was reverse-transcribed into first-strand complementary DNA, using Moloney-murine leukaemia virus-reverse transcriptase (M-MLV-RT) and oligo(dT)12–18 primer (Invitrogen). Quantitative real-time PCR of the common part of the short and long form of human leptin receptor transcript was carried out on an ABI PRISM 7900 HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using specific FAM-labelled probe and primers (prevalidated TaqMan Gene expression Assay, Applied Biosystems). Leptin receptor gene expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control gene. Relative quantitation of gene expression was calculated with the comparative CT method (2^{-CT})[15] and was plotted as fold-change compared to untreated cells, that were chosen as the reference sample.

2.5. *Binding of recombinant human leptin to A549 cells*

In order to assess the exogenous recombinant human leptin binding to its receptor, the cells cultured in presence or absence of apigenin and of leptin, alone or combined, were incubated for 1 h at 4 °C with the recombinant human leptin (0.5 μM). Cells were then washed in cold PBS and incubated for 1 h at 4 °C with the anti-leptin antibody Ob. Non-immune IgG at the same titre as the primary antibody was used as a negative control. After washing, the FITC-conjugated polyclonal swine anti-rabbit Ig were added for 30 min at 4 °C to the cells before flow cytometric analysis.

2.6. *Clonogenic assay*

The colony growth of A549 cells exposed to apigenin and to leptin, alone or combined, was assessed. In 35-mm Petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) a lower layer was prepared using complete RPMI 1640 medium in 0.5% agarose. The cells were harvested and seeded (5×10^4) on the upper layer with 0.3% agarose prepared with the same medium as the lower layer, and finally incubated for 21 days at 37 °C in an atmosphere containing 0.5% CO_2. At the end of incubation, colonies were counted under an inverted phase-contrast microscope (Leitz, Wetzlar, Germany). The experiment was conducted in triplicate. Colonies were defined as cell aggregates with at least 40 cells. The true number of colonies was calculated as the number of aggregates on the positive control subtracted from the number of colonies on the experimental plates. The results are expressed as percentages of the control value.

2.7. *Analysis of intracellular reactive oxygen species (ROS)*

Intracellular ROS were measured by the conversion of the non-fluorescent dichlorofluorescein diacetate (DCFH-DA; Sigma–Aldrich, Milan, Italy) in a highly fluorescent compound, DCF, by monitoring the cellular esterase activity in the presence of peroxides in A549 cells. The ROS generation in A549 cell exposed to apigenin and to leptin, alone or combined, was assessed by uptake of 1 μM DCFHDA, incubation for 10 min at room temperature in the dark, followed by flow cytometric analysis.

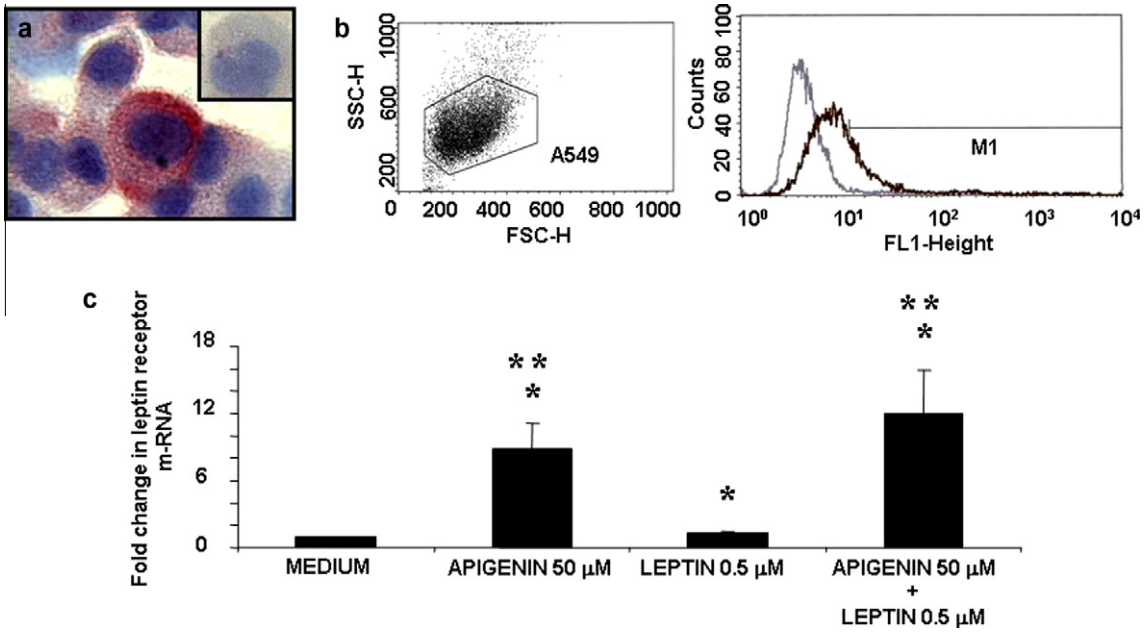

Fig. 1 – A549 cells express leptin receptors. (a) Immunocytochemistry from A549 cells for leptin receptors. Corner: negative control. Magnification at 1000×. (b) Flow-cytometry analysis for leptin receptor expression in A549 cells. Physical parameters (left side) and overlay of fluorescence intensity (FL_1) (right side) related to expression of leptin receptor versus events histogram of cells cultured in medium. Grey peak: negative control. (c). Leptin receptor m-RNA expression is quantified by real-time reverse transcription-PCR assays. GAPDH gene is used for normalisation. Data are expressed as fold induction over baseline condition. Significantly difference *versus medium and **versus leptin. $P < .05$, unpaired *t*-test.

2.8. Determination of cell death and apoptosis

The death and apoptosis of A549 cell exposed to apigenin and to leptin, alone or combined, was assessed by uptake of 1 μM ethidium bromide and by Annexin V test based on redistribution of phosphatidylserine (PS) on cell surface[6] respectively, followed by flow cytometric analysis.

2.9. Pleural fluid collection

Pleural fluids (PF) were collected from patients with lung adenocarcinoma ($n = 6$, age range 45–78 years). All subjects gave informed written consent and the study was approved by the institutional review board for human studies and was consistent with Helsinki Declaration. Malignant effusions were defined as exudates associated with a pathologic diagnosis of cancer from cytological examination of pleural fluids and from pathologic examination of lung tissues. No patients were undergoing anti-inflammatory or steroid therapies. The fluids were drawn into polypropylene bags containing heparin (10–20 IU/ml) and were subsequently centrifuged at 400*g* for 10 min. Cell-free fluids were immediately frozen at −70 °C until they were used in subsequent experiments.

2.10. Statistical analysis

Results are expressed as means ± SD. Unpaired *t*-test was performed. A *P*-value of less than .05 was considered statistically significant.

3. Results

3.1. Constitutive leptin receptor protein expression by A549 cells

In our *in vitro* model, we first tested, by immunocytochemistry analysis and by flow cytometry, whether A549 cells constitutively expressed leptin receptor proteins. A549 cells expressed leptin receptor proteins (Fig. 1a and b) demonstrating that these cells are sensitive to leptin biological effects.

3.2. Effect of apigenin and of leptin on leptin receptor mRNA

To investigate the modulation of leptin receptor in A549 cells, the effect of apigenin and of leptin, alone or combined, on leptin receptor mRNA expression was tested. We observed that A549 cells constitutively express mRNA for the common

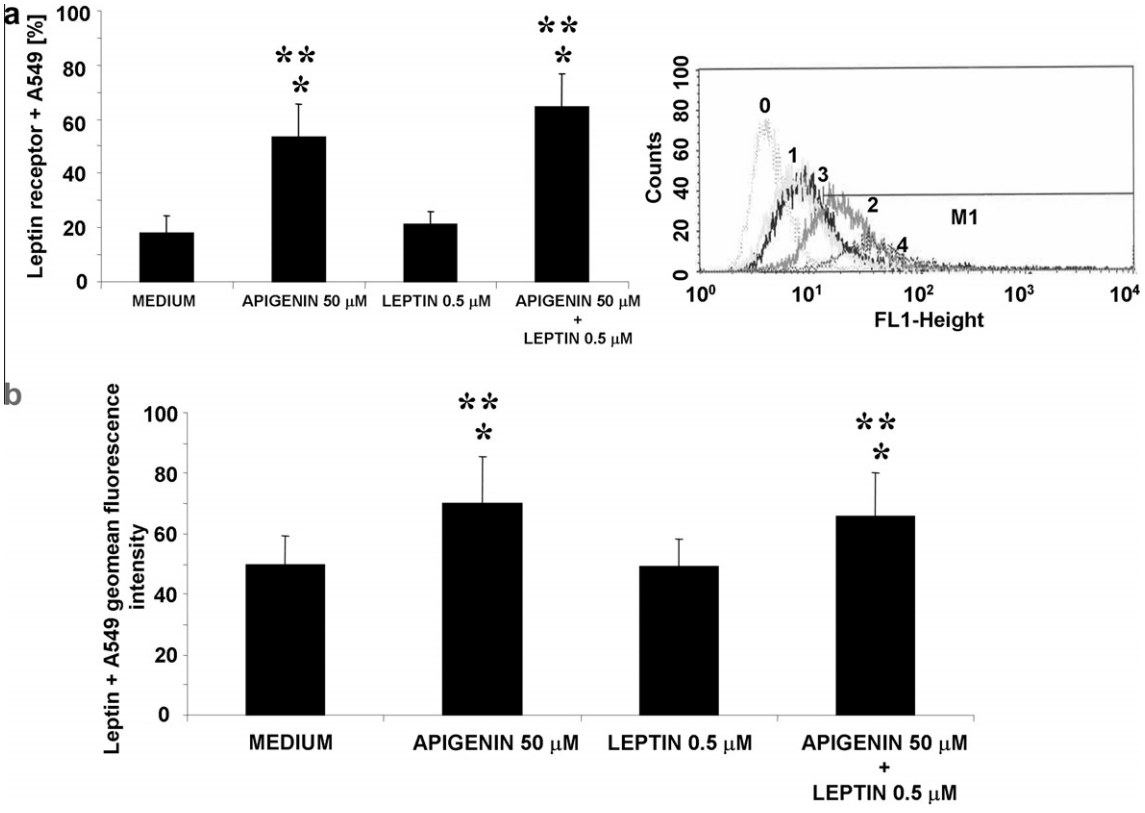

Fig. 2 – Apigenin and apigenin combined with leptin affect leptin receptor and leptin protein expression. (a) Leptin receptor protein expression is significantly increased by apigenin and by apigenin combined with leptin *versus medium and **versus leptin. Right, representative example of flow cytometric analysis: 0, negative control; 1, medium; 2, apigenin; 3, leptin; 4, apigenin combined with leptin. (b) Leptin protein synthesis is significantly increased by apigenin and by apigenin combined with leptin *versus medium and **versus leptin. $P < .05$, unpaired *t*-test.

part of the short and the long isoform of leptin receptor and that both apigenin and leptin stimulation, alone or combined, significantly increased the mRNA expression of the leptin receptors ($p < .005$, $p < .0002$, $p < .008$) (Fig. 1c). Also, the mRNA expression of the leptin receptors was significantly higher in A549 cells cultured with apigenin alone or combined with leptin ($p < .006$ and $p < .009$, respectively) than in A549 cells cultured with leptin (Fig. 1c).

3.3. *Effect of apigenin and of leptin on leptin receptor and leptin proteins*

The effect of apigenin and of leptin, alone or combined, was explored on leptin/leptin receptor pathway by investigating the leptin receptor and the leptin protein expressions by flow cytometry. Apigenin alone or combined with leptin significantly increased the constitutive leptin receptor ($p < .003$ and $p < .002$, respectively) (Fig. 2a) and constitutive leptin expressions ($p < .03$ and $p < .05$, respectively) (Fig. 2b). Furthermore, apigenin alone or combined with leptin significantly increased the leptin receptor ($p < .003$ and $p < .002$, respectively) and leptin expressions ($p < .02$ and $p < .04$, respectively) than A549 cells cultured with leptin (Fig. 2a and b).

3.4. *Detection of leptin binding in A549 cells*

To further get inside the leptin/leptin receptor pathway activation, we tested the ability of exogenous recombinant human leptin to bind A549 cells, in presence or absence of apigenin and of leptin, alone or combined. We found that significantly higher levels of the leptin binding were detected in A549 cells cultured with apigenin alone or combined with leptin ($p < .009$ and $p < .0004$, respectively) than in A549 cultured in medium or cultured with leptin ($p < .03$ and $p < .003$, respectively) (Fig. 3).

3.5. *Effect of apigenin and of leptin on A549 cell proliferation*

Because apigenin promotes anti-proliferative role in various malignant cell lines[5] and leptin delivers proliferative signals in bronchial epithelial cell line[11] we investigated whether apigenin delays and leptin induces cell proliferation (assessed by colony numbers). Lower colony number was found in A549 cells cultured with apigenin alone or combined with leptin in comparison to medium alone ($p < .002$ and $p < .009$, respectively) or in comparison to leptin cultured cells ($p < .001$ and $p < .002$, respectively) (Fig. 4) and significantly higher colony number was found in A549 cells cultured with leptin in comparison to medium ($p < 0.02$) (Fig. 4).

3.6. *Effect of apigenin and of leptin on A549 intracellular ROS generation*

Some evidences suggest that both apigenin and leptin are able to generate free radical and oxidative damage that affect apoptosis and cell proliferation in several cytotypes.[5,12] Based on this, we have also investigated the ability of apigenin and

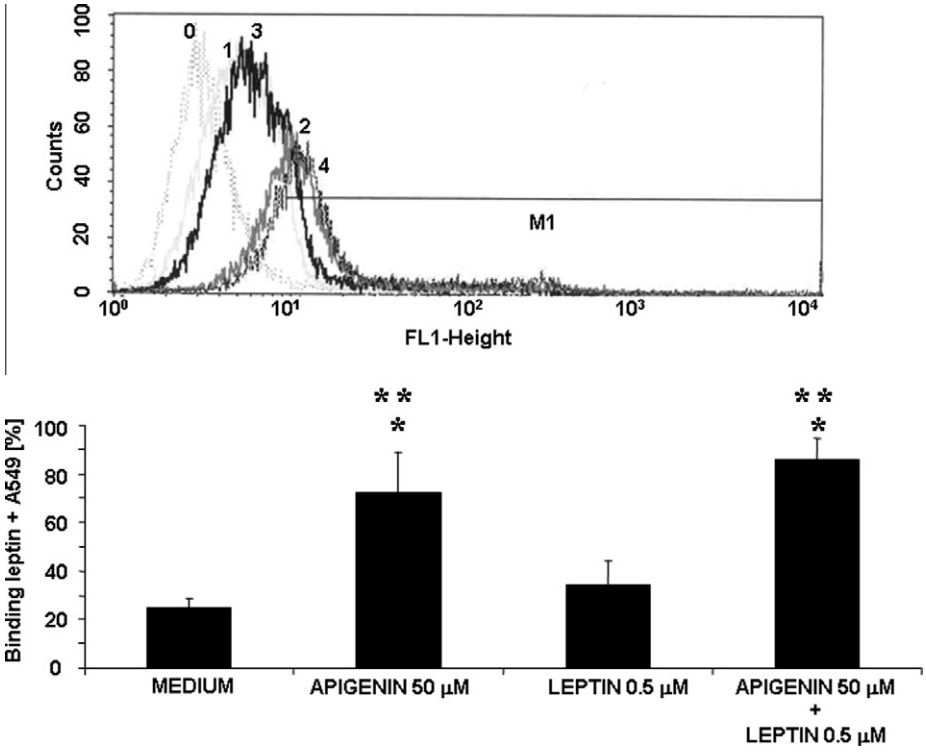

Fig. 3 – Apigenin and apigenin combined with leptin affect leptin binding. Leptin binding is significantly increased by apigenin and by apigenin combined with leptin *versus medium and **versus leptin. Upper, representative example of flow cytometric analysis: 0, negative control; 1, medium; 2, apigenin; 3, leptin; 4, apigenin combined with leptin. P < .05, unpaired t-test.

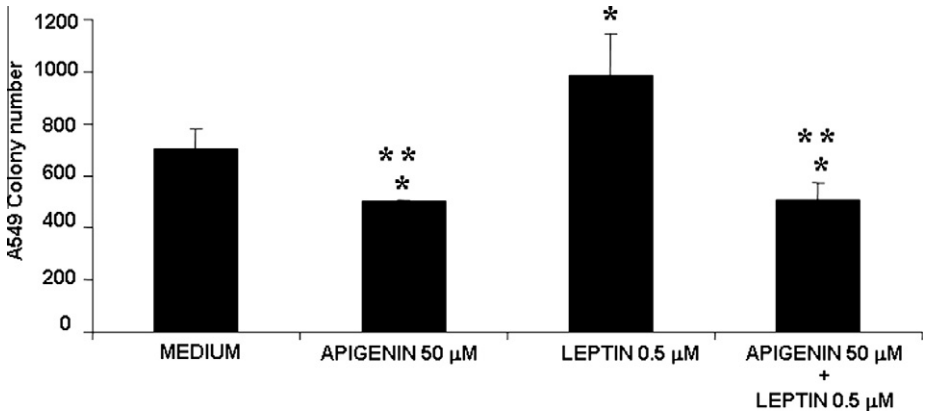

Fig. 4 – Apigenin and leptin affect A549 cell proliferation. The colony number is significantly decreased by apigenin and by apigenin combined with leptin and it is significantly increased by leptin. Significantly difference *versus medium and **versus leptin. P < .05, unpaired t-test.

leptin, alone or combined, to induce ROS generation in A549 cells. We found that apigenin alone or combined with leptin significantly increased ($p < .0002$ and $p < .001$, respectively) the spontaneous release of ROS (Fig. 5), whereas leptin did not.

3.7. *Effect of apigenin and of leptin on cell death and cell apoptosis*

Since it has been assessed that apigenin induces apoptosis in malignant cells[5] and leptin has anti-apoptotic activity in inflammatory cells,[6,10] we assessed whether apigenin induces and leptin delays cell death and apoptosis in A549 cells. With regard to cell death, apigenin alone or combined with leptin significantly increased ($p < .0006$ and $p < .0009$, respectively) whereas leptin significantly decreased ($p < .05$) the spontaneous cell death in a time-dependent manner (Fig. 6). Furthermore, A549 cells cultured with apigenin alone or combined with leptin showed a significantly higher cell death ($p < .0001$ and $p < .0004$, respectively) than A549 cells cultured with leptin (Fig. 6). With regard to apoptosis, apigenin alone or combined with leptin significantly increased the spontaneous

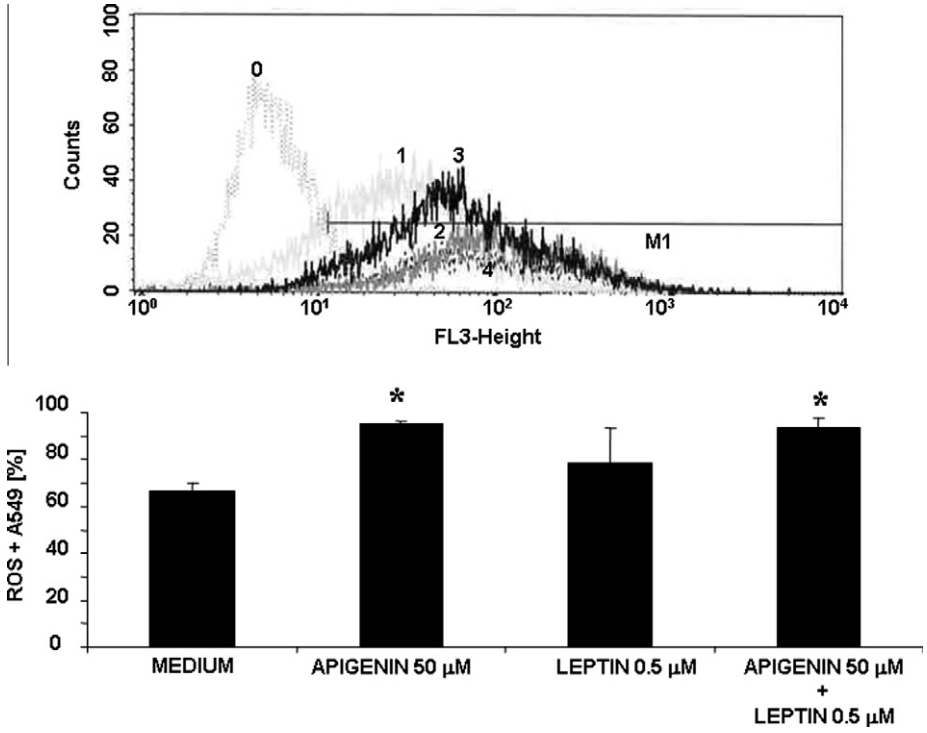

Fig. 5 – Apigenin and apigenin combined with leptin affect intracellular ROS generation. Intracellular ROS generation is significantly increased by apigenin and by apigenin combined with leptin *versus medium. Upper, representative example of flow cytometric analysis: 0, negative control; 1, medium; 2, apigenin; 3, leptin; 4, apigenin combined with leptin. P < .05, unpaired t-test.

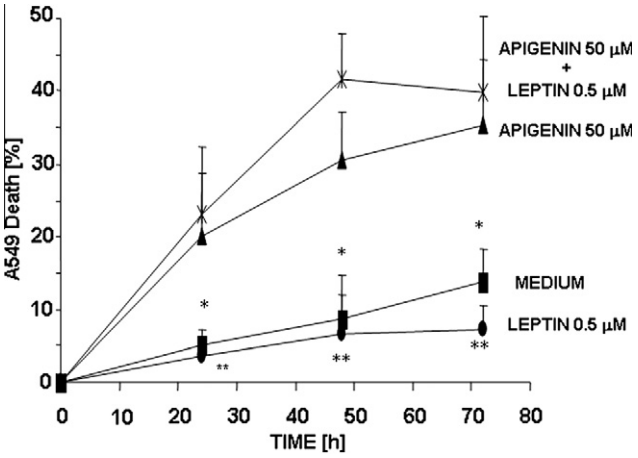

Fig. 6 – Apigenin and leptin affect A549 cell death. Cell death is significantly increased by apigenin and by apigenin combined with leptin and significantly decreased by leptin. Significantly difference *versus apigenin, apigenin combined with leptin and leptin and **versus apigenin and apigenin combined with leptin. P < .05, unpaired t-test.

redistribution of PS in A549 cultured in medium ($p < .02$ and $p < .03$, respectively) or cultured with leptin ($p < .009$ and $p < .02$, respectively) (Fig. 7). Since lung adenocarcinoma PF are protective for cancer cells,[16,17] we investigated whether apigenin and leptin, alone or combined, affect A549 cell apoptosis also in the presence of lung adenocarcinoma PF. We observed that also in the presence of lung adenocarcinoma PF, apigenin, alone or combined with leptin, significantly increased the spontaneous redistribution of PS in A549 cultured in medium ($p < .04$ and $p < .0009$, respectively) or cultured with leptin ($p < .05$ and $p < .004$, respectively) (Fig. 7). Furthermore, in comparison to medium alone apigenin, alone or combined with leptin, induced a higher PS redistribution ($p < .05$) in the absence than in the presence of lung adenocarcinoma PF (Fig. 7).

4. Discussion

Apigenin and leptin have been implicated in the proliferation and in the apoptotic pathway of several types of cancer cells. Apigenin promotes tumour suppression[5,18] while leptin favours tumour progression[7–9] and modulates the innate and adaptive immune responses.[13] The role of these two molecules, alone or combined, in the lung cancer has never been explored, thus we investigated whether apigenin delays and leptin induces cell proliferation in lung adenocarcinoma.

In the present study we demonstrate for the first time that apigenin interferes, *in vitro*, with the effects of leptin on the proliferation, cell death and apoptosis of the lung adenocarcinoma cell line A549. In addition, the apigenin effects seem to be dominant on the leptin mediated effects since they persist also when the cells are cultured with apigenin combined with leptin.

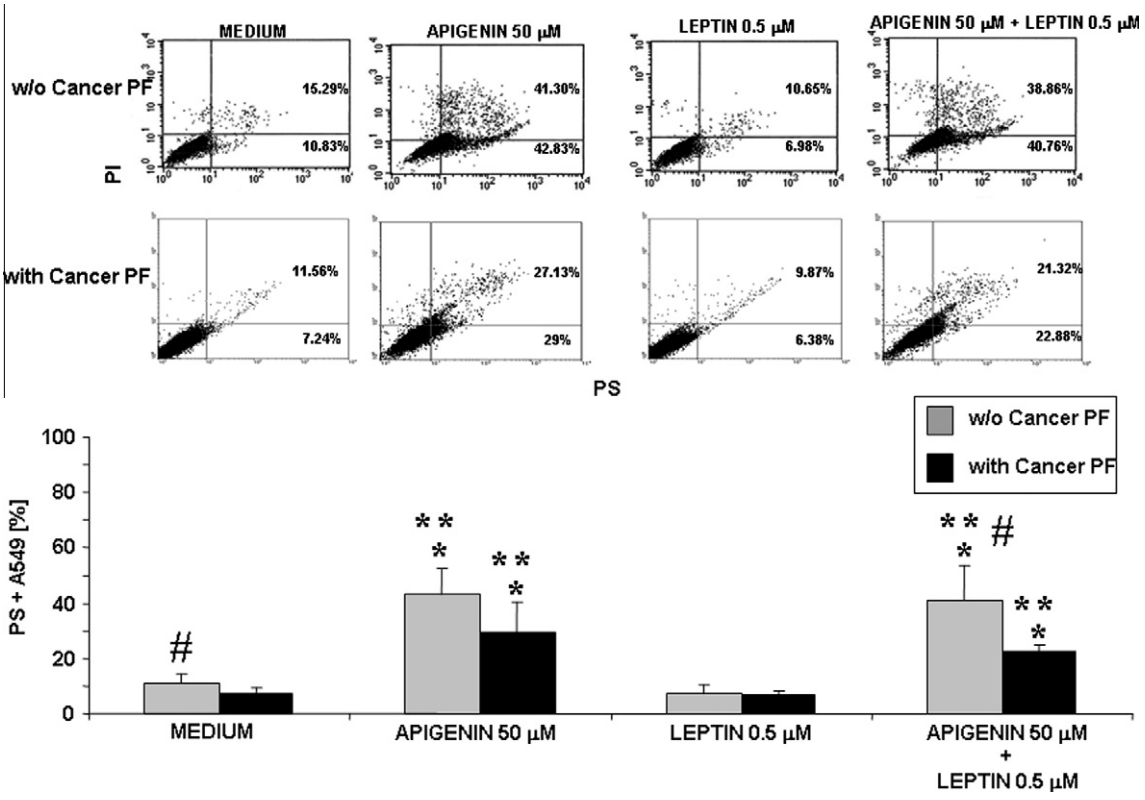

Fig. 7 – Apigenin and apigenin combined with leptin affect cell apoptosis. Apoptosis is significantly increased by apigenin and by apigenin combined with leptin *versus medium and **versus leptin. # Cell apoptosis is significantly reduced in the presence of lung adenocarcinoma PF. Upper, representative example of flow cytometric analysis. The numbers indicate the percentage of apoptotic and necrotic cells, respectively. P < .05, unpaired t-test.

First, leptin receptor is expressed by A549 cells. Second, apigenin is able to significantly increase the synthesis of both leptin receptors and leptin other than the leptin binding activity. Third, apigenin significantly decreases cell proliferation and significantly increases cell death whereas leptin has the opposite effect. Fourth, apigenin significantly increases spontaneous apoptosis either in absence or in presence of adenocarcinoma pleural fluids (PF). Fifth, apigenin significantly increases ROS release whereas leptin does not. In all these events, apigenin shows to have the strongest effect in comparison to leptin on A549 cells. On the other hand leptin, when combined with apigenin, is not able to counteract the role of apigenin at all. To the best of our knowledge, this is the first *in vitro* study that evaluates the role of apigenin and of leptin, alone or combined, on cell proliferation, cell death and apoptosis in A549 cells. Apigenin is a flavonoid involved in lung cancer biology. A recent study shows that apigenin induces decreased percentage of viable cells, cytochrome *c* release and caspase-3, -8 and -9 activation, leading to apoptosis in a dose- and time-dependently manner in A549 cells.[19] Furthermore, *in vivo* experiments assessed that the number of tumour cells adhering to lung vessels was significantly diminished in mice treated with a single dose of apigenin, indicating the ability of apigenin in the inhibition of tumour cell metastasis and substantiating the role of the intravascular processes in the metastatic cascade.[20] On the other hand, it is well known that leptin has, both *in vivo* and *in vitro*, a positive correlation with increased risk of several cancers as well as proliferative and anti-apoptotic effects on cancer cell cultures *in vitro*.[8,9] In the human lung, the leptin/leptin receptor pathway has been shown to be upregulated in the submucosa of COPD patients, where leptin is identified as cytokine-like hormone with a proliferative role for immune cells.[10] Also, the leptin/leptin receptor pathway is involved in lung epithelial homeostasis and proliferation and its downregulation is positively correlated with airway remodelling in asthma.[11] In addition, recent clinical observations report that higher serum leptin levels seem to represent an additional, independent risk factor for NSCLC whereas plant foods consumption shows a protective association.[7] In our *in vitro* model, leptin significantly increases cell proliferation and significantly decreases cell death. Thus, leptin in lung cancer play a role in epithelial homeostasis activity together with growth-promoting effects. To further get inside the leptin/leptin receptor pathway expression and activation in the lung adenocarcinoma, we tested the ability of exogenous recombinant human leptin to bind the A549 cells. Interestingly, in our study we found that apigenin is able to strongly increase, alone or combined with leptin, not only the expression of leptin receptors and the leptin synthesis but also the leptin binding. This is in line with a recent experimental study on 3T3-L1 pre-adipocyte differentiation where apigenin causes unexpectedly enhanced leptin synthesis.[21] Based on these evidences, we suppose that both apigenin and leptin may compete for a common signal intracellular pathway. Recently, it has been suggested that part of the chemo-preventive activity of apigenin may be mediated by its ability to modulate the mitogen-activated protein kinase (MAPK) cascades[22] and that the treatment with apigenin is accompanied by an increase in ROS and phosphorylation of the MAPKs (ERK1/2)[23] in fibroblast-like cells.

The effects of apigenin on MAPK cascades activation and its cellular responses appear to be complex depending on cell type. Activation of MAPKs during apigenin-induced apoptosis is further confirmed by the use of MAPK pharmacological inhibitors, as SB203580 (p38 MAPK inhibitor).[23] On the other hand, the leptin receptors are known to activate MAPK cascades after leptin binding.[6,11] Also for leptin activity, the use of SB203580 confirmed that MAPK cascades are involved in transducing leptin mediated anti-apoptotic and proliferative signalling, either in immune cells[6] and in bronchial epithelial cells.[11] MAPK cascades are involved in the signalling of various cellular responses such as proliferation and inflammation and also cell death, where, in some cell types, the activation of MAPK cascades is extremely important for induction of cellular death, growth arrest and apoptosis.[24,25] Therefore, we show that apigenin increases the leptin/leptin receptor pathway and the leptin binding activity leading to negatively interfere with leptin apoptotic activity in the lung adenocarcinoma cell line A549. All these events mediated by apigenin and by recombinant human leptin are associated with a similar activation of MAPKs (data not shown). Based on our observation, we sought to demonstrate that different activity of apigenin and of leptin on ROS production is responsible for the observed differences on survival effects in the lung adenocarcinoma cell line A549. With regard to oxidative stress, some evidences suggest that both apigenin and leptin are able to generate free radical and oxidative damage in several cytotypes[5,12] that affect apoptosis and cell proliferation. ROS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems. In cancer, the regulatory mechanisms of cell growth and proliferation mediated by flavonoids is widely complex: while low level of ROS can stimulate the cell division and the promotion of tumour growth, a high level of ROS is cytotoxic to the cell and can induce apoptosis or even necrosis.[26] ROS can act in each of the stages of carcinogenesis.[27] This latter is a multistage process characterised by cumulative action of multiple events (initiation, promotion and progression).[28] Indeed, despite the presence of the cell's antioxidant defence system to counteract oxidative damage from ROS, oxidative damage has been proposed to play a key role in the development of cancer. Actually, for this potentiality to increase oxidative stress in cancer cells, flavonoid activity has recently been investigated in a large amount of *in vitro* studies.[5,22,26,29] Based on this, we have also investigated the ability of apigenin and of leptin, alone or combined, to induce ROS generation in A549 cells. Our results showed that apigenin, and not leptin, significantly increases intracellular ROS generation. Furthermore, we assessed that apigenin significantly decreases cell proliferation and increases both cell death and apoptosis by increasing intracellular ROS generation, as previously demonstrated in human in leukaemia cells,[5] in prostate cancer cells and in liver cancer cells.[18] Apigenin belongs to the flavonoid family and it is well known that it may exert both an anti-oxidant role (antioxidant ROS scavenging)[30] or pro-oxidant role (pro-oxidant ROS producing).[23] This latter activity has been shown to lead to apoptosis, as previously reported on A549

lung adenocarcinoma cell line.[29] Consistently, in the present study, we assessed that apigenin increased both the pro-oxidant and the pro-apoptotic activities in A549 cells. Finally, experiments were performed to assess whether the anti-cancer effects of apigenin persist in a milieu promoting cancer growth (i.e. malignant pleural effusions). Specific cancers, such as lung cancer or cancer of the breast, ovary and stomach preferentially metastatise the pleural compartment. Pleural inflammation generates an ideal micro-environment to attract cancer cells, to promote their growth and their protection. In particular, PGE2 present in malignant effusions contributes to cancer expansion and may protect cancer cells by anti-proliferative effects induced by talc.[17] Furthermore, pleural lymphocytes from cancer patients have a higher intracellular CD94/NKG2A expression and exhibit a reduced cytotoxic activity against cancer cells when compared to autologous peripheral blood T lymphocytes and to T lymphocytes isolated from congestive heart failure pleural fluids.[16] Since lung adenocarcinoma pleural fluids (PF) are protective for cancer cells,[16,17] we investigated whether apigenin and leptin affect A549 cell apoptosis in the presence or in the absence of PF from cancer patients. First, the effect in increasing cell apoptosis is higher in the absence than in the presence of lung adenocarcinoma PF both in presence and absence of apigenin and leptin, alone or combined. Second, apigenin alone or combined with leptin, significantly increases the spontaneous adenocarcinoma cell line A549 apoptosis both in the presence or in the absence of PF, further supporting the potent anti-cancer activity of apigenin. Further studies *in vivo* are needed to confirm this new host protective role of apigenin in the therapeutic approach of lung cancer.

5. Conclusions

In summary, we provide evidence that both apigenin and leptin play a role in the survival of the lung adenocarcinoma cell line A549. In this context, the apigenin mediated activities abolish and reverse the proliferative and the anti-death roles promoted by leptin, despite leptin appearing to be a strong mediator for cancer cell proliferation and survival. Apigenin activity also persists in the presence of milieu promoting cancer growth as lung adenocarcinoma pleural fluids. In this scenario apigenin acts as a potent antagonist of pro-carcinogenic activities of leptin and it may represent a useful supplement in the therapy of patients with lung adenocarcinoma.

Authors' contributions

BA contributed study design, cultured cells, performed most experiments, carried out flow cytometry analysis and wrote the article. SL contributed RT-PCR and clonogenic assay was carried out. GS contributed cells culture and performed most experiments followed by flow cytometry analysis. FM contributed immunocytochemistry and flow cytometry analysis was carried out. CP contributed study design, critical insights, reviewed the article. GM contributed to conception of the study and critical insights. GM contributed data interpretation and critical insights. PE contributed study design, evaluated results, edited and reviewed the manuscript. All authors have read and approved the final manuscript.

Conflict of interest statement

None declared.

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