ING4 Inhibits Proliferation and Induces Apoptosis in Human Melanoma A375 Cells via the Fas/Caspase-8 Apoptosis Pathway

Yanli Ma, Xue Cheng, Fei Wang, Jisheng Pan, Jing Liu, Hongxiao Chen

Yongchen Wang, Limin Cai

Department of Dermatology, First Affiliated Hospital of Harbin Medical University, and Department of Dermatology, Second Affiliated Hospital of Harbin Medical University, Harbin, and Department of Dermatology, Chinese People’s Liberation Army 404st Hospital, Weihai, PR China

Introduction

Cutaneous melanoma is a common malignant skin cancer and its incidence is increasing steeply [1]. Melanomas have a poor prognosis because they are highly invasive and metastasize rapidly; in addition, they are resistant to various conventional therapies. The molecular mechanisms underlying melanoma development are complex and not very clear.

Many researchers believed that the inhibitor of growth (ING) family, which is generally composed of five members in mammals (ING1, ING2, ING3, ING4 and ING5), may be involved in tumorigenesis and melanoma development. They are thought to participate in various biological activities, including cell cycle arrest, DNA damage response and senescence, gene transcriptional regulation, signal transduction and apoptosis [2, 3].

Studies showed that ING5 could enhance Fas-induced apoptosis in an INCA1-dependent manner [4]. ING1 may contribute to trichostatin A-induced apoptosis in p53-deficient glioblastoma cells by regulating the Fas/caspase-3 apoptosis pathway [5]. Furthermore, ING3-
mediated UV-induced apoptosis via the Fas/caspase-8 pathway was reported in human melanoma cells [6]. As a tumor suppressor, ING4 could influence apoptosis of malignant cells through multiple ways, including the Fas/FasL pathway. In previous studies, ad-ING4 plus had an additive effect to chemotherapy with cisplatin on the abnormal expression of apoptosis-related proteins and subsequent activation of the Fas-mediated intrinsic and extrinsic apoptotic pathways [7]. Ad-ING4 combined with radiation induced apoptosis by regulating Fas, FasL, cleaved caspase-3, Bax and Bcl-2 in human non-small cell lung cancers [8]. However, it was not clear if ING4 could affect melanoma progression by regulating the Fas-mediated apoptosis pathway.

In our previous research, ING4 expression was downregulated in melanoma tissue; in addition, it could partly suppress tumor growth and enhance apoptosis in a human melanoma cell line (M14) by mitochondrial-mediated apoptosis [9]. However, the role of ING4 in melanomas has been scarcely elucidated. It remains to be clari-
fied whether other mechanisms are also implicated in the inhibition of melanoma growth and induction of cell apoptosis by ING4 regulation. In this study, A375, a human melanoma cell line, was used to explore the universal effects of ING4 on melanoma cells; besides, we further investigated the correlation between ING4 expression and melanoma growth and apoptosis as well as the activation of the Fas/caspase-8 apoptosis pathway in vitro and in vivo by up-regulating ING4 expression.

Materials and Methods

For further details, see the online supplementary materials (for all online suppl. material, see www.karger.com/doi/10.1159/000444050) (fig. 1; suppl. fig. 1–4)

Results

ING4 Protein Expression in Transfected A375 Cells

Western blot analysis showed that ING4 protein expression was significantly increased in A375/pLenO-GTP-ING4 cells compared with A375 and A375/pLenO-GTP cells (fig. 2).

Effect of ING4 Protein on the Proliferation of A375 Cells in vitro

In the three study groups, growth of A375 cells varied (online suppl. fig. 1). The growth rate of the A375/pLenO-GTP-ING4 group was lower than that of the A375 and A375/pLenO-GTP groups, which showed a similar growth rate. Viability of A375 cells did not differ in the three groups. As shown in table 1, the optical density (OD) value of A375/pLenO-GTP-ING4 was significantly lower than that of A375 (p = 0.000) and A375/pLenO-GTP (p = 0.000) on days 2–5 (day 1: p > 0.05). OD was not different between A375 cells and A375/pLenO-GTP (p > 0.05) cells on days 1–5.

Effect of ING4 Protein Expression on A375 Cell Apoptosis in vitro

Annexin-V FITC analysis by flow cytometry revealed that the percentage of apoptotic cells in early stage in A375/pLenO-GTP-ING4 (7.733 ± 0.5400%) was higher than that of A375 (5.8267 ± 0.2914%) and A375/pLenO-GTP (5.8967 ± 0.1266%; p = 0.001; online suppl. fig. 2). Similar results were also obtained by TUNEL assay. For visualization by DAB staining, positive cells displayed brown staining in the nucleus with nuclear fragmentation, and no staining was negative. As shown in table 2 and online supplementary figure 3, more positive cells were observed in A375/pLenO-GTP-ING4 cells compared with A375 and A375/pLenO-GTP cells. The apoptotic index (AI) of A375/pLenO-GTP-ING4 cells (36.60 ± 5.41%) was markedly higher than that of A375 (12.60 ± 1.52%) and A375/pLenO-GTP (5.80 ± 1.48%; p = 0.001; online suppl. fig. 2). These results revealed that high expression of ING4 could enhance apoptosis in A375 cells in vitro.

Inhibitory Effect of ING4 on the Growth of Xenografts in Nude Mice in vivo

The weight and volume of xenografts in three kinds of nude mice are listed in table 3 (see also online suppl.

### Table 1. OD values (x10^-1) of the three groups of A375 cells after culture for 1–5 days

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1 mean ± SD</th>
<th>Day 2 mean ± SD</th>
<th>Day 3 mean ± SD</th>
<th>Day 4 mean ± SD</th>
<th>Day 5 mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375/pLenO-GTP-ING4</td>
<td>1.51 ± 0.078</td>
<td>1.58 ± 0.077</td>
<td>2.00 ± 0.091</td>
<td>3.27 ± 0.168</td>
<td>4.56 ± 0.088</td>
</tr>
<tr>
<td>A375/pLenO-GTP</td>
<td>1.67 ± 0.108</td>
<td>2.07 ± 0.035</td>
<td>4.08 ± 0.0690</td>
<td>7.23 ± 0.0710</td>
<td>8.66 ± 0.143</td>
</tr>
<tr>
<td>A375</td>
<td>1.66 ± 0.0500</td>
<td>2.04 ± 0.0610</td>
<td>4.13 ± 0.0640</td>
<td>7.34 ± 0.262</td>
<td>8.67 ± 0.0940</td>
</tr>
</tbody>
</table>

Statistical differences were analyzed by one-way ANOVA. The OD value of A375/pLenO-GTP-ING4 was significantly decreased after transfection (p = 0.000 vs. A375 and A375/pLenO-GTP on days 2–5). No difference was observed between A375 or A375/pLenO-GTP (p > 0.05) on days 1–5.

### Table 2. AI of the three groups of A375 cells in vitro and the xenograft tissue in vivo (means ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell culture</th>
<th>Xenograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375/pLenO-GTP-ING4</td>
<td>36.60 ± 5.41</td>
<td>42.80 ± 9.23</td>
</tr>
<tr>
<td>A375/pLenO-GTP</td>
<td>14.20 ± 1.64</td>
<td>6.60 ± 1.52</td>
</tr>
<tr>
<td>A375</td>
<td>12.60 ± 1.52</td>
<td>5.80 ± 1.48</td>
</tr>
</tbody>
</table>

* p = 0.000, b p = 0.003, vs. A375 and A375/pLenO-GTP (one-way ANOVA). No difference was observed between A375 and A375/pLenO-GTP (p > 0.05).
Xenografts of A375/pLenO-GTP-ING4 were significantly smaller than those of A375 and A375/pLenO-GTP both in weight (p = 0.000) and volume (p = 0.007 and p = 0.002). No statistical difference was found in the weight and volume of xenografts between A375 and A375/pLenO-GTP cells (p > 0.05).

Xenograft tissues were cut into 5-μm sections. In HE staining, tumor tissue presented a nest-like growth pattern. Tumor cells were epithelioid with clear or hypochromatic cytoplasm and trachychromatic atypical nucleus (fig. 3). Pathological karyokinesis was a rare occurrence and almost without patch necrosis in the xenograft tissue of the A375/pLenO-GTP-ING4 group, while pathological karyokinesis and patch necrosis were more frequent in the other two groups. As shown in figure 3 and table 2 by TUNEL assay, there were more apoptotic cells in the xenograft tissue of the A375/pLenO-GTP-ING4 group.

Table 3. The weight and volume of nude mouse xenografts from the three groups of A375 cells (means ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight, g</th>
<th>Volume, mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375/pLenO-GTP-ING4</td>
<td>0.230±0.035</td>
<td>49.136±21.767</td>
</tr>
<tr>
<td>A375/pLenO-GTP</td>
<td>0.354±0.037</td>
<td>242.450±51.029</td>
</tr>
<tr>
<td>A375</td>
<td>0.392±0.033</td>
<td>271.082±72.859</td>
</tr>
</tbody>
</table>

n = 5/group. In the A375/pLenO-GTP-ING4 group, the weight (p = 0.000) and the volume of xenografts were decreased vs. the A375 and A375/pLenO-GTP groups (p = 0.007 and p = 0.002, respectively). No difference was observed between the A375 and A375/pLenO-GTP groups (p > 0.05). One-way ANOVA was employed.

Fig. 3. Ultrathin sections of nude mouse xenograft tissues were cut and stained with HE (a; ×100) and assessed by TUNEL (b; ×400).

Table 3. The weight and volume of nude mouse xenografts from the three groups of A375 cells (means ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight, g</th>
<th>Volume, mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375/pLenO-GTP-ING4</td>
<td>0.230±0.035</td>
<td>49.136±21.767</td>
</tr>
<tr>
<td>A375/pLenO-GTP</td>
<td>0.354±0.037</td>
<td>242.450±51.029</td>
</tr>
<tr>
<td>A375</td>
<td>0.392±0.033</td>
<td>271.082±72.859</td>
</tr>
</tbody>
</table>

n = 5/group. In the A375/pLenO-GTP-ING4 group, the weight (p = 0.000) and the volume of xenografts were decreased vs. the A375 and A375/pLenO-GTP groups (p = 0.007 and p = 0.002, respectively). No difference was observed between the A375 and A375/pLenO-GTP groups (p > 0.05). One-way ANOVA was employed.

Fig. 4. Expression of ING4 and Fas/FasL system proteins, including Fas, FasL, caspase-8, caspase-3 and PARP, in cancerous tissues of nude mouse xenografts by immunohistochemistry (×100).

(For figure see next page.)
ING4 and Fas/Caspase-8 in Human Melanoma

Dermatology 2016;232:265–272
DOI: 10.1159/000444050

A375 A375/pLenO-GTP A375/pLenO-GTP-ING4

ING4
FasL
Fas
Caspase-8
Caspase-3
PARP
group (AI: 42.80 ± 9.23%) than the A375 (AI: 5.80 ± 1.48%) and A375/pLenO-GTP groups (AI: 6.60 ± 1.52%; p = 0.003). There was no statistical difference between the A375 and A375/pLenO-GTP groups (p > 0.05).

Furthermore, a markedly increased expression of ING4 in the xenograft tissue of the A375/pLenO-GTP-ING4 group, and lower expression levels of ING4 in the A375 and A375/pLenO-GTP groups were observed by immunohistochemistry (fig. 4).

**Effect of ING4 on the Expression of Fas/Caspase-8 Pathway Proteins in A375 Cells**

In vitro, several Fas/caspase-8 pathway proteins, including Fas, FasL, caspase-8, caspase-3 and pro-caspase-3 acid repetitive protein (PARP), were detected in the three groups of A375 cells by Western blotting. Expression of caspase-8 and caspase-3 was up-regulated, and FasL and PARP expression was down-regulated in the A375/pLenO-GTP-ING4 group compared with the A375 and A375/pLenO-GTP groups (fig. 2). In vivo, these proteins were assessed in the xenograft tissue of nude mice from the three groups of A375 cells by immunohistochemistry. Consistent with the data from Western blotting, expression of Fas, caspase-8 and caspase-3 was increased and expression of FasL and PARP was decreased in the xenograft tissues of the A375/pLenO-GTP-ING4 group compared with those in the A375 and A375/pLenO-GTP groups (fig. 4).

**Discussion**

The incidence of melanoma, one of the most aggressive cancer types in humans, is steadily increasing worldwide [10]. However, the complex molecular mechanisms involved in tumorigenesis still remain largely unknown. In melanomas, ING4 expression was decreased compared with several types of nevi [9, 11]. Moreover, ING4 was found to inhibit angiogenesis in melanomas [12], and migration and invasion of melanoma cells in humans [13]. Therefore, the correlation between ING4 expression and melanoma cell proliferation and apoptosis was studied.

Previous studies demonstrated that ING4 expression was dramatically decreased in many cancers, including glioma [14], hepatocellular carcinoma [15], breast cancer [16], head and neck carcinoma [17], and gastric carcinoma [18]. In vitro, we found that the rate of growth and viability of melanoma A375 cells transfected with exogenous ING4 gene were markedly decreased, and more cells presented apoptosis than nontransfected A375 cells. The results suggested that the growth rate of A375 cells was negatively associated with ING4 expression, with slower growth rate and cell viability accompanied by higher ING4 expression. The same results were obtained from experiments in vivo. In the group showing ING4 overexpression, the weight and volume of xenografts of nude mice were significantly lower than those of nontransfected A375 mice. The TUNEL assay showed that more apoptotic cells were observed in xenograft samples from the group with ING4 overexpression. The above results indicated that ING4 overexpression could remarkably inhibit the proliferation and growth of melanoma cells and induce their apoptosis.

In our previous research, ING4 induced apoptosis in melanoma M14 cells via a mitochondrial apoptosis pathway mediated by Bcl-2/Bax/Cyt-c [9], but ING4 may also be involved in other apoptosis pathways in melanomas. Several studies demonstrated that INGs could enhance apoptosis via the death receptor-mediated extrinsic pathway (Fas/caspase-8 apoptosis pathway) in different cancers [19, 20]. Our in vitro and in vivo data indicate that ING4 overexpression could increase the expression of Fas, an important member of the tumor necrosis factor receptor superfamily, which is involved in apoptosis. Fas-mediated apoptosis is triggered by FasL, which subsequently leads to the recruitment of FADD (Fas-associated death domain), which further recruits and cleaves procaspase-8. Activated caspase-8 can activate effectors such as caspase-3, which subsequently cleaves PARP to cause cell apoptosis [21–23]. It was demonstrated that low expression of Fas and high expression of FasL were a frequent occurrence in melanoma [24], and the Fas-mediated apoptosis pathway was thought to be implicated in apoptosis of melanoma cells. Based on the above information, we hypothesized that ING4 could regulate the proliferation and apoptosis of malignant melanoma cells by the Fas/caspase-8 apoptosis pathway.

In addition, tumors expressing FasL are immune privileged since they can counterattack natural killer cells and tumor-reactive cytotoxic T lymphocytes. Consequently, these tumors are able to evade immune destruction [25, 26]. Thus, melanoma cells with up-regulated FasL expression usually have the ability to kill Fas-bearing activated lymphocytes adjacent to melanoma cells to escape immune surveillance [27]. In our study, the expression level of Fas paralleled that of ING4 expression, whereas the expression level of FasL was negatively correlated with that of ING4. We speculated that the low expression of ING4 and the loss of its functions might help melanoma

Dermatology 2016;232:265–272
DOI: 10.1159/000444050

Ma/Cheng/Wang/Pan/Liu/Chen/Wang/Cai
In conclusion, ING4 is able to play antitumor roles in melanomas via the Fas/caspase-8 apoptosis pathway, e.g. to inhibit proliferation and growth of melanoma cells and induce apoptosis. The results of this study suggest that ING4 may be involved in mechanisms affecting the immune surveillance of melanoma cells. Nevertheless, we did not analyze the correlation of ING4 and Fas/caspase-8 pathway protein expression in tissue from patients by immunohistochemistry or other methods, which might present a limitation of this study. Future studies are needed to confirm and extend our results on the effect of ING4 on melanoma cells, particularly with respect to their ability to escape immune surveillance.

Acknowledgments

This study (project No. 81072234) was supported by the National Natural Science Foundation of China. We are grateful to all those who participated in the experiments, data analysis and in the writing for this study.

Statement of Ethics

This study was approved by the Experimental Animal Ethics Committee of Harbin Medical University.

Disclosure Statement

The authors have no conflicts of interest to disclose.

References


13. Li J, Li G: Cell cycle regulator ING4 is a suppressor of melanomaangiogenesis that is reg- ulated by the metastasis suppressor BRMS1. Cancer Res 2010;70:10445–10453.


IN4 and Fas/Caspase-8 in Human Melanoma

Dermatology 2016;232:265–272
DOI: 10.1159/000444050

271


