Table I. Primer pairs for real-time PCR.

Genes	Accession No.	Primer sequences (5' to 3')
PAX1	AL035562	Forward: CACCCCCGCAGTGAATG Reverse: CGCCCACGGCAGAGA
PAX2	AH006910	Forward: TGGTCTGGACTTTAAGAGATGTGTGTCT Reverse: GAAGGTGTCAGCTCGCAAGTG
PAX3	AH003224	Forward: CCTCTTACCAGCCCACATCTATTC Reverse: CGTGCTTTGGTGTACAGTGCTT
PAX4	AB008913	Forward: GGCACTGGAGAAAGAGTTCC Reverse: CTTGAGCTTCTCTTGCCGAC
PAX5	M96944	Forward: TGTCAGGCCCTGCGACAT Reverse: GGCGACCTTTGGTTTGGAT
PAX6	M77844	Forward: GGAAGCTGCAAAGAAATAGAACATC Reverse: TTCTCGGGCAAACACATCTG
PAX7	X96743	Forward: GCCACAGCTTCTCCAGCTACTC Reverse: ATGCTCATCACCTGAGGAGACA
PAX8	L19606	Forward: TGAGGGCGTCTGTGACAATG Reverse: AGCTGTCCATAGGGAGGTTGAA
PAX9	BC001159	Forward: GAATGCAGGCAGCCAGAGA Reverse: GGAGACGGAATTTCCCATCA
β-actin	XM037235	Forward: TTGCCGACAGGATGCAGAA Reverse: GCCGATCCACACGGAGTACT

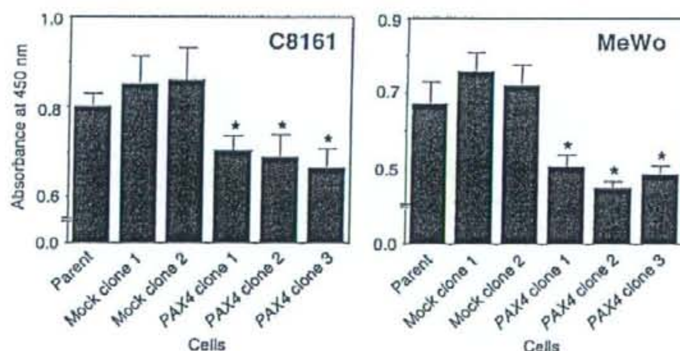

Figure 4. DNA synthesis of *PAX4*-overexpressing cell lines and control cell lines. DNA synthesis was evaluated by BrdU incorporation assay. There was a statistically significant difference between each of the *PAX4*-overexpressing cell lines (*PAX4* clone 1, 2 and 3) and each of the control cell lines (parent, Mock clone 1 and 2) by one-way ANOVA followed by Scheffe's *F* analysis as a post-hoc test ($p<0.01$).

Treatment of melanoma cells with 5-azacytidine, a DNA demethylating agent or trichostatin A, a histone deacetylase inhibitor. Cells were plated in 100-mm tissue culture dishes at a cell density of 1×10^6/dish. After 24 h, the cells were treated with 5 μM 5-azacytidine (Sigma, St. Louis, MO) for 2 days, or with 1 to 100 nM trichostatin A (Sigma) for 2 to 5 days.

Results

Expression levels of 9 PAX genes in human melanoma specimens, melanoma cell lines and nevus specimens. We first analysed the expression levels of 9 types of *PAX* genes in 16 melanoma specimens, 7 melanoma cell lines and 5 nevus specimens by a real-time RT-PCR method. Of the 9 *PAX* genes, 3 *PAX* genes such as *PAX3*, *PAX4* and *PAX9* were expressed in nevus tissues. As shown in Fig. 1, the expression levels of *PAX4* and *PAX9* in the melanoma specimens and melanoma cell lines were significantly low compared to those in the nevus specimens ($p<0.01$, Mann-Whitney U test). None of the melanoma cell lines showed expression of the two *PAX* genes.

Overexpression of PAX4 in human melanoma C8161 and MeWo cells transfected with the PAX4 expression vector. As

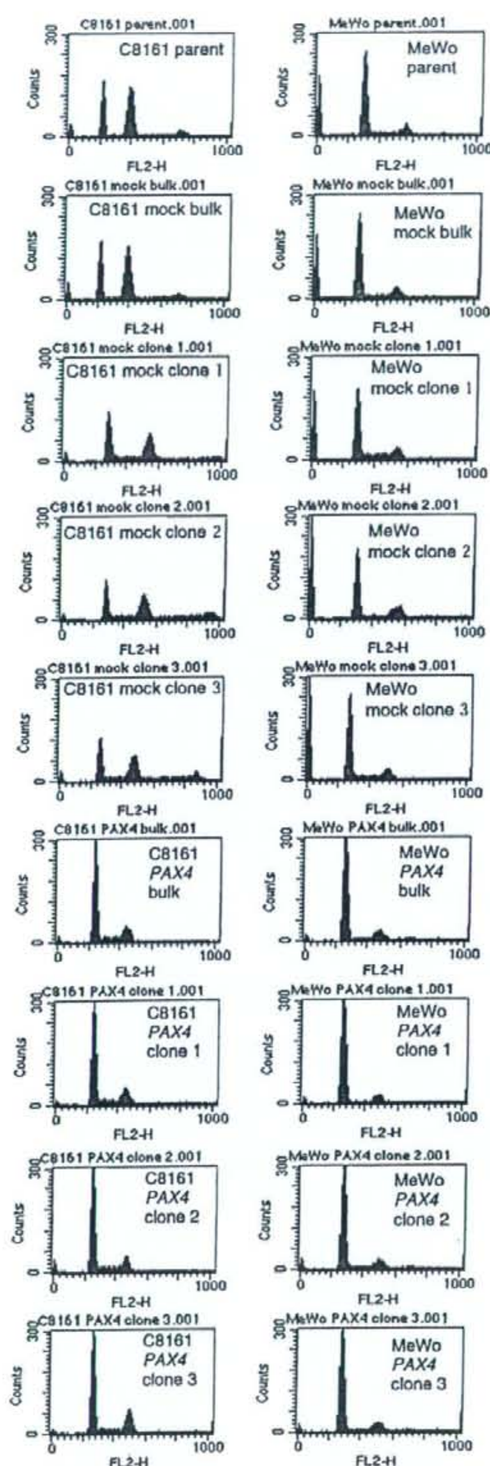

Figure 5. Cell cycle analysis of *PAX4*-overexpressing cell lines and control cell lines. Cell cycle analysis was performed by detecting fluorescence intensity of the cells stained with propidium iodide with flow cytometry. Left panel,data of C8161 cells; right panel, MeWo cells.

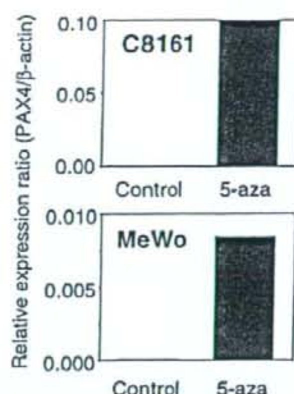

Figure 6. Induction of *PAX4* expression by treatment with 5-azacytidine (5-aza), a demethylating agent. RNA was extracted from the cells treated with 5 μM 5-aza for 2 days and *PAX4* expression was analysed by real-time RT-PCR method.

the expression of *PAX4* was lost or low in melanoma tissues and melanoma cell lines compared to nevus tissues, we speculated that *PAX4* functioned as a tumor suppressor. To examine whether the expression of *PAX4* influences the proliferative behaviour of melanoma cells, we stably transfected C8161 and MeWo cells with a *PAX4* expression vector, pCDNA3. 1HisA-*PAX4*. The cell lines were transfected with empty pCDNA3.1HisA (mock transfectants) as a control. RNA was extracted from each parent line and transfected sublines and subjected to the analysis of expression of *PAX4* by a real-time RT-PCR method. In the C8161 and MeWo cell lines, all the cloned cell lines transfected with *PAX4* and *PAX4*-transfected cell lines before cell cloning showed higher expression of *PAX4* than the parent and mock-transfected cells (Fig. 2A and B). Immunoblot analysis also revealed that *PAX4*-transfected cells expressed the PAX4 protein (Fig. 2C).

Decreased cell growth of PAX4-overexpressing melanoma cells. We examined the influence of *PAX4*-overexpression on cell growth of melanoma cells by WST-8 assay. As shown in Fig. 3A, the growth of all the C8161 cell lines overexpressing *PAX4* was significantly lower than the control cell lines (parent and Mock-transfectants) after incubation for 93.5 h ($p<0.01$). In MeWo cells, the growth of all the cell lines overexpressing *PAX4* was significantly lower than any of the control cell lines after incubation for 89 h ($p<0.01$). The inhibitory effect of *PAX4*-overexpression on the cell growth was more prominent in MeWo cells compared to C8161 cells.

Decrease in DNA synthesis in melanoma cells by PAX4-overexpression. To examine whether the growth inhibition by *PAX4*-overexpression was due to a decrease in DNA synthesis, we performed a BrdU-uptake assay. As shown in Fig. 4A and B, the activity of DNA synthesis diminished by *PAX4*-overexpression in C8161 and MeWo cells.

Increase in G0/G1 phase fraction of cell cycle in melanoma cells by PAX4-overexpression. We then analysed cell cycle

distribution by flow cytometry in order to clarify whether decreased DNA synthesis by *PAX4*-overexpression was related to cell cycle arrest. In C8161 and MeWo cells, PAX4-overexpression extended the G0/G1 phase fraction and reduced S or G2/M phase fraction (Fig. 5), while no essential changes were detected in the Mock transfectants as compared to the parent cells.

Induction of PAX4 expression by treatment with 5-azacytidine. We tested whether the silencing of the *PAX4* gene in C8161 and MeWo cells was due to the DNA methylation or histone deacetylation. When the two cell lines were treated with 5 μM of the demethylating agent 5-azacytidine for 2 days, they expressed the *PAX4* gene (Fig. 6). However, treatment with histone deacethylase inhibitor trichostatin A did not induce the expression of the *PAX4* gene in any of the cell lines (data not shown).

Discussion

Nine members have been identified in the human *PAX* gene family. They are classified into 4 groups based on the structural domains other than the paired domain (25). The first group (group III: *PAX3* and *PAX7*) contains an octapeptide motif and a homeodomain; second one (group II: *PAX2*, *PAX5* and *PAX8*) contains an octapeptide motif and a truncated homeodomain; third one (group IV: *PAX4* and *PAX6*) or fourth one (group I: *PAX1* and *PAX9*) contains only a homeodomain or an octapeptide motif. So far, there are many studies indicating that *PAX* genes of group II and III contribute to oncogenesis. These *PAX* genes are often highly expressed in a variety of tumors: *PAX2* in prostate cancer (26), *PAX5* in medulloblastoma (14), *PAX8* in ovarian cancer (17) and *PAX3* and *PAX7* in Ewing sarcoma (12,27). Furthermore, experimental analyses also showed oncogenic functions of these *PAX* genes: Silencing of *PAX2* by antisense oligonucleotides or siRNA suppressed the growth of renal cancer cells (28) or induced apoptosis in ovarian and bladder cancer cells (17); mouse *Pax1*, *2*, *3*, *6* and *8 in vitro* transformed NIH 3T3 cells and the transformants formed tumors in mice (16); *PAX2*, *5* and *8* inhibited transactivation of a p53-responsive reporter in culture cells (21).

However, there are few studies on anti-oncogenic functions of *PAX* genes; for example, *PAX2* and *PAX8* are able to transactivate WT1, one of the tumor suppressor genes and thus they may be considered to work tumor-suppressively (19,29). A low level of *PAX6* expression in malignant astrocytic gliomas correlates with unfavourable outcomes (30) and overexpression of *PAX6* suppresses cell growth due to G1 arrest of cell cycle in human glioma cells (31).

Here, we showed for the first time that *PAX4* potentially functioned as a tumor suppressor in human melanoma cells. Namely, the expression levels of *PAX4* were significantly low in melanoma tissues compared to nevus tissues. Furthermore, forced expression of *PAX4* suppressed the growth of melanoma cells, mainly due to a decrease in DNA synthesis through cell cycle arrest at the G1 phase. This phenomenon is similar to the growth inhibition of glioma cells by *PAX6*, as mentioned above (31). It is notable that *PAX4* has common features with *PAX6*. Both belong to the same group (group IV) of the *PAX* family and are required for the normal development of pancreas, especially hormone-producing endocrine cells (32). Additionally, they are capable of binding a common element in the glucagons, insulin and somatostatin promoters (33,34). It is unlikely that regulatory functions of the transcription of these genes related to the pancreas development work as tumor suppressors. However, it may be reasonable to think that *PAX4* as well as *PAX6* affects the expression levels of or interactions with molecules associated with the control system of the cell cycle, not as yet characterized. Further studies should help to identify the molecules targeted by *PAX4*.

How is the expression of *PAX4* suppressed in melanoma? The expression levels of *PAX4* in C8161 and MeWo were induced by treatment with 5-azacytidine, a DNA-demethylating agent but not TSA, a histone deacethylase inhibitor. No deletion of the *PAX4* gene in these cell lines was detected by genomic DNA analysis (unpublished data). Futhermore, the deletion of chromosome 7q on which the *PAX4* gene was localized was not detected although its amplification was reported (35,36). Thus, the suppression of *PAX4* in melanoma is likely to be due to DNA-methylation although we need to identify the methylated sites.

This is the first study suggesting that *PAX4* potentially functions as a tumor suppressor. Although it is necessary that further studies reveal detailed molecular mechanisms by which *PAX4* works as a tumor suppressor, *PAX4* may provide an important avenue for a therapeutic strategy to melanoma.

Acknowledgements

We thank Dr Takahide Miyamoto for the generous gift of the human *PAX4* expression vector and Ms. Masako Yanome for her help in the preparation of the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research (B) to Y.Y. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Journal of Plastic, Reconstructive & Aesthetic Surgery (2008) xx, 1–5

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JPRAS
An International Journal of Surgical Reconstruction
www.JPRASurg.com

Merkel cell carcinoma of the face: an analysis of 16 cases in the Japanese

Akira Saito, Arata Tsutsumida, Hiroshi Furukawa, Noriko Saito, William Mol, Mitsuru Sekido, Satoru Sasaki, Kohei Oashi, Chu Kimura, Yuhei Yamamoto*

Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan

Received 20 December 2007; accepted 15 March 2008

KEYWORDS
Merkel cell carcinoma;
Staging system;
Japanese;
Face

Summary *Background:* There is no agreement regarding a staging system and optimal treatment of Merkel cell carcinoma. Some centres have reported results from larger series of patients, but these do not include Asian or Japanese centres.
Objective: The purpose of this study was to retrospectively review our experience with the surgical treatment of MCC of the face in the Japanese and to study its management and outcome using the staging system described by Clark et al.
Methods: We report our experiences with 16 cases between 1991 and 2004. Patients and tumour characteristics, treatment variables and outcome were analysed.
Results: The follow-up periods ranged from 1 to 180 months. The average was 32.6 months and the median was 17.5 months. The relapse-free survival for all patients was 51% at 2 years. The relapse-free survival was 80% for the patients with Stage I and 33% with Stage II at 2 years.
Conclusion: This staging system was suggested to reflect prognosis although the number of patients in this series was small. Sentinel lymph node biopsy should be considered to determine the accurate nodal staging, and patients with MCC of the head and neck may be treated according to the revised staging system by Clark et al.
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Merkel cell carcinoma (MCC), first described in 1972 by Toker,[1] is a rare and highly malignant tumour of the skin. MCC cells are neuroendocrine cells present in the basal layer of the epidermis and are presumed to be the cell of origin of the cancer.[2] MCC occurs in sun-exposed areas, with approximately 50% of cases arising in the head and neck region.[3] MCC occurs in elderly patients with an average age at diagnosis of 69 years; only 5% of cases occur before the age of 50 years.[4]

* Corresponding author. Tel.: +81 11 706 6978; fax: +81 11 706 7827.
E-mail address: yu-h1@med.hokudai.ac.jp (Y. Yamamoto).

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doi:10.1016/j.bjps.2008.03.027

Please cite this article in press as: Saito A et al., Merkel cell carcinoma of the face: an analysis of 16 cases in the Japanese, J Plast Reconstr Aesthet Surg (2008), doi:10.1016/j.bjps.2008.03.027

By use of data from the US Surveillance, Epidemiology, and End Results (SEER) Program, the estimated incidence is 0.23 per 100 000 people in Caucasian populations.[4] The incidence in Negroids (0.01 cases per 100 000) as well as in Polynesians seems to be lower. No analysis of changing incidence trends for MCC has been reported.[5]

While relevant literature is often limited to small descriptive studies,[6–8] some centres have reported results from larger series of patients.[9–15] This group included the experience of some US,[9,10] European[11] and Australian centres,[12–15] but did not include that of Asian or Japanese centres.

There is no specific staging system for MCC but a commonly adopted system (MSKCC system) divides patients into three groups according to whether they have local disease (Stage I), locoregional disease (Stage II), or distant disease (Stage III) (Table 1).[16,17] Stage I is sub-divided into two groups, according to the tumour diameter (Stage IA; $\leq$2 cm, Stage IB; >2 cm). This staging system is simple and allows the development of a logical management protocol appropriate to the stage,[17] but Yiengpruksawan et al. suggested that the tumour size was not predictive of local recurrence or development of lymph node metastasis.[16]

Clark et al. have recently described a revised staging system for MCC in a general cohort that appears to be more predictive and better at stratifying outcome (Table 2).[3] In this staging system, T classification is further sub-divided by tumour size less than 1 cm (T1) or larger than 1 cm in maximal diameter (T2) and N classification is sub-divided by the number of involved regional lymph nodes, with negative lymph nodes (N0), less than two positive lymph nodes (N1) or more than two positive lymph nodes (N2).

The purpose of this study was to retrospectively review our experience with the surgical treatment of MCC involving the facial region in the Japanese and to study its management and outcome using the revised staging system described by Clark et al.

Materials and methods

We have reviewed 16 cases of Merkel cell carcinoma in the face that were treated at Hokkaido University Hospital and Hakodate Central General Hospital over a 13-year period from 1991 to 2004. Medical information was obtained from the patients' medical records and telephone follow ups. The tumour characteristics were classified according to the site and the size of the primary lesion, and the stage. Treatment variables analysed include excision margins and reconstructive procedure. Tumours were staged using the staging system proposed by Clark et al.[3]

Table 1 Staging system for Merkel cell carcinoma[16]

Stage	Description
IA	Disease confined to skin and $\leq$2 cm in diameter
IB	Disease confined to skin and >2 cm in diameter
II	Involvement of regional lymph nodes
III	Metastatic disease

Results

Patients and tumour characteristics (Table 3)

Of the 16 patients, there were four males (25%) and 12 females (75%). Their ages ranged from 41 to 100 years (median 83.5 years). The most common site of tumour location was the cheek (69%), followed by upper eyelid (13%), lower eyelid (6%), nose (6%), and upper lip (6%). The average tumour size at initial presentation was 1.82 cm (range from 0.4 to 6.5 cm). Staging was ascertained in 15 patients. Stage I disease was present in five patients (33%), Stage II in eight patients (53%), and Stage III in two patients (13%). All patients in Stage II were classified into Stage IIb.

Primary treatment (Table 4)

All patients received surgery as a primary treatment modality. Therapeutic lymph node dissection was performed in one patient and elective lymph node dissection in one patient.

The excision margins ranged from 0.5 to 2 cm (mean 1.06 cm). Out of 11 patients who presented with cheek tumours, 10 received excision with 1 cm or more excisional margins. Out of five patients who presented with tumours elsewhere other than the cheek, four patients received excision with margins of less than 1 cm. Among the five patients in Stage I, one patient was excised with margins of less than 1 cm while four patients had excision with margins of 1 cm or more. Among eight patients in Stage IIb, five patients had excision with margins of 1 cm or more and three patients had excision with margins of less than 1 cm. Adjuvant radiotherapy was administered to the tumour bed in only one patient (stage I).

Reconstruction (Table 4)

Some patients required reconstructive surgery following primary excision. Among the patients who presented with tumours on the cheek, four had wound closure with a split-thickness skin graft (STSG) and three patients had closure using a free flap. Apart from the patients with cheek tumours, one patient had closure with a local flap and the remainder had closure using a combination of a local flap and a cartilage graft.

Outcome

The follow-up periods ranged from 1 to 180 months. The average was 32.6 months and the median was 17.5 months.

No patients with Stage I disease had local recurrence. Among eight patients with Stage II disease, one of three patients with a surgical margin of less than 1 cm had local recurrence while the other two patients had nodal recurrence. Among two patients with Stage III disease, one had nodal recurrence and both of them developed distant metastasis. There was only one tumour-related death in the whole sample, but there were four non-tumour-related deaths.

The relapse-free survival for all patients was 51% at 2 years and 41% at 5 years (Figure 1). The relapse-free

Table 2 Revised staging system for Merkel cell carcinoma[3]

T	N	M	Stage
T1, primary tumour ≤ 1 cm	N0, negative regional lymph nodes	M0, no evidence of distant metastatic disease	I, T1, N0, M0
T2, primary tumour > 1 cm	N1, ≤2 positive regional lymph nodes	M1, distant metastases present	IIa, T1, N1, M0
	N1, >2 positive regional lymph nodes		IIb, T2, N0, M0 III, Any T, N2, M0 IV, Any T, Any T, M1

survival was 80% for patients with Stage I and 33% with Stage II disease at 2 years (Figure 2).

Discussion

Yienpruksawan et al. described a staging system for Merkel cell carcinoma in 1991,[16] and it is now adopted commonly.[17] They also reported that Stage I patients had longer 5-year survival than Stage II patients and sub-classification of Stage I was not predictive of local recurrence and development of lymph node metastasis when stage I was divided into two groups according to tumour diameter (Stage IA; ≤2 cm, Stage IB; >2 cm).[16] Some reported that a tumour size >2 cm had a poor prognosis,[10,18,19] while others reported the size of the tumour did not affect the outcome,[9,20,21] similar to Yienpruksawan et al. Clark et al. proposed the revised staging system for MCC. The border of T classification was changed to 1 cm and they reported that primary tumour size >1 cm was a poor prognostic predictor for disease—specific survival and overall survival.[3] In our series, there was a significant difference in relapse-free survival between the stages. Although the number of patients was small, these data suggested that the staging system described by Clark could be a prognostic factor.

Table 3 Patient demographics and characteristics

Characteristic	No.	%
Age, years		
Median	83.5	
Range	41–100	
Sex		
Male	4	25
Female	12	75
Size, cm		
Median	17.5	
Range	4–65	
Location		
Cheek	11	69
Upper eyelid	2	13
Lower eyelid	1	6
Nose	1	6
Upper lip	1	6
Stage		
I	5	33
II	8	53
IIa	0	0
IIb	8	53
III	2	13
IV	0	0

Wide local excision was advocated for the treatment of early stage MCC, although there are controversies regarding the necessary margins.[22,23] Margins of 2 to 3 cm have been generally recommended as optimal.[16,24,25] However, the location of many lesions, particularly in the head and neck region, makes the achievement of 2–3 cm margins often impractical from a cosmetic and functional perspective.[26] In our series, wide excision margins (>2 cm) were not achieved in the head and neck, similar to some other reports.[9,23,26] The lesions were divided into two groups according to the location of primary tumour; cheek or other sub-unit (eyelid, nose and lip). The surgical margins for the tumours located on the cheek were larger than for the other sub-units (cheek, 1.2 cm; other sub-unit, 0.6 cm). This suggested that the surgical margins were restricted by the anatomical sub-unit in the face. The reconstructive options following primary excisions included STSG and free flap for the tumours on the cheek in many cases and local flaps on other sub-units. This suggested that the reconstructive procedures were due to the site and the size of the defect followed by excision.

Several authors reported that surgical margins did not predict the outcome.[10,27,28] Clark et al. described that this negative finding is important in the head and neck, since achieving wide or even negative margins may be difficult without mutilating surgery.[3] However, the question remains of what the recommended margins are in the head and neck region. Within Stage IIb patients in our series, we found local recurrence in one patient and lymph node metastases in two patients with margins <1 cm but none with margins >1 cm. We recommend wide excision of the primary lesion with a margin at least 1 cm for early stage MCC in the head and neck. Even if this is difficult, a negative surgical margin should be achieved.

Table 4 Surgical margin and reconstructive options according to location

Location	Surgical margin	No.	Reconstructive surgery				
			Suture	STSG	Local flap	Local flap, cartilage graft	Free flap
Cheek	<10 mm	1	1	0	0	0	0
	≥10 mm	10	1	4	2	0	3
Others	<10 mm	4	0	0	1	3	0
	≥10 mm	1	0	0	0	1	0

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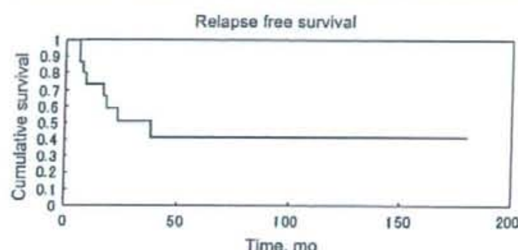

Figure 1 The relapse-free survival for all patients was 51% at 2 years and 41% at 5 years.

MCC is considered a radiosensitive cancer.[13] Leonard et al. found MCC more radiosensitive than small-cell carcinoma of the lung, malignant melanoma, or malignant glioma.[29] Several clinical studies have suggested that the risk of local recurrence and regional lymph node metastases may be significantly lower in patients who received adjuvant radiotherapy, and many studies have recommended locoregional radiotherapy to reduce local recurrence.[12,27,30–32] However, some studies which showed the effectiveness of radiotherapy, did not take account of T classification.[12,28,32] There is a question of whether adjuvant radiotherapy should be considered routinely in all patients of MCC. Some reports suggested that the use of adjuvant radiotherapy was associated with improved survival, particularly in larger tumours.[19,33] If, however, primary tumors are classified according to a more detailed staging system, the outcome observed with surgery might be the same as the outcome observed with adjuvant radiotherapy in patients with smaller tumours.

MCC has similarities to small cell carcinoma of the lung, both histologically and structurally, therefore similar regimens are usually used for MCC patients.[23] Some authors suggested that MCC is a chemosensitive disease,[34–36] and the addition of systemic chemotherapy to local excision and radiation is a reasonable treatment option because many patients die of distant metastasis.[37] In contrast, it has been reported that the use of adjuvant chemotherapy was not associated with survival.[38,39] The role of routine adjuvant chemotherapy has remained controversial and should be further investigated with new protocols in the future.

Clark et al. discussed treatment options for MCC according to the disease stages produced by themselves.[3] They recommended surgery plus adjuvant irradiation in both revised Stage II and Stage III disease. Otherwise, they suggested surgery alone in patients with revised Stage I disease because of their low risk of death from the disease.[3] In our series, there were no local recurrences, lymph node metastases, or distant metastases with Stage I. The sample size was not large enough to determine the optimal therapeutic approach in terms of disease stage; however, it may be possible to omit the adjuvant irradiation in the patients with revised Stage I disease.

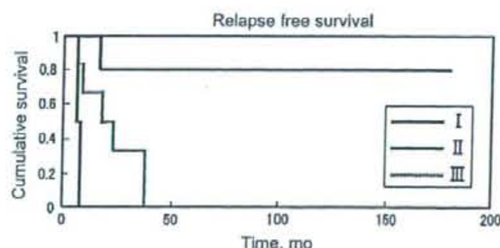

Figure 2 The relapse-free survival was 80% for patients with Stage I, 33% with Stage II at 2 years according to the revised staging system by Clark et al.

The presence of lymph node involvement is an important prognostic factor.[12,31,40,41] Sentinel lymph node biopsy (SLNB) is a well-known staging strategy for melanoma.[42] Although we performed SLNB for only two patients of MCC in this series, the use of SLNB has been also reported in MCC recently.[11,39,43–45] Maza et al. reported that they performed SLNB in 23 patients for whom no nodal disease found on physical examination and micrometastatic disease was found in 11 patients (47.8%).[11] Gupta et al. reported that they performed SLNB in 122 patients with clinically negative lymph node involvement and the findings revealed nodal involvement in 39 cases (32%). Patients with a positive SLNB who received adjuvant nodal therapy had a relapse-free survival rate of 51% at 3 years compared with 0% for patients who did not receive nodal therapy. They concluded that SLNB is important for both prognosis and therapy, and should be performed routinely for patients with MCC.[43] Allen et al. suggest the importance of determining nodal status and they do not recommend the routine use of adjuvant radiotherapy in MCC patients who have undergone SLNB and showed no micrometastatic disease.[39] We consider that SLNB should be performed if possible and we should choose optimal treatments according to a more accurate staging system.

MCC is an aggressive carcinoma characterised by high rates of early locoregional relapse and distant failure,[27] and many authors recommend multimodal treatment including wide local excision, radiotherapy and chemotherapy.[9,17,24,27,35,41,46,47] We advocate wide local excision with 1 cm margins for the treatment of MCC of the face. For Stage I patients, we recommend wide local excision without adjuvant therapy. For Stage IIa patients, we recommend wide local excision, neck dissection, and adjuvant radiotherapy. For Stage IIb patients, we recommend wide local excision and adjuvant radiotherapy. For Stage III patients, we recommend a combination of wide local excision, neck dissection and adjuvant radiotherapy, and chemotherapy should be used unless contraindicated. For Stage IV patients, chemotherapy may be used.

In conclusion, a simple staging system proposed by Yiengpruksawan has been adopted by several investigators for MCC. In the present study, we reviewed our experiences of 16 patients with MCC of the face using a revised staging system devised by Clark et al., and this staging system was suggested to reflect prognosis. In our opinion, SLNB should be considered to determine the accurate nodal staging, and patients with MCC of the head and neck may be treated according to the revised staging system by Clark et al. In our study, the number of patients was small and the design was retrospective, so the evidence level was not high. In the future, we need a perspective study with a larger number of cases to analyse MCC disease.

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Please cite this article in press as: Saito A et al., Merkel cell carcinoma of the face: an analysis of 16 cases in the Japanese, J Plast Reconstr Aesthet Surg (2008), doi:10.1016/j.bjps.2008.03.027

Simvastatin inhibits growth via apoptosis and the induction of cell cycle arrest in human melanoma cells

Akira Saito, Noriko Saito, William Mol, Hiroshi Furukawa, Arata tsutsumida, Akihiko Oyama, Mitsuru Sekido, Satoru Sasaki and Yuhei Yamamoto

Competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (the statins) that inhibit the synthesis of mevalonic acid are in wide use for treatment of hypercholesterolemia. Although antitumor effects on a variety of cell types have been reported for statins, the effect of simvastatin (one of the statins) on human melanoma cell lines is not known. Here, we report antitumor effects of simvastatin on human melanoma cell lines. We treated human melanoma cell lines, A375M, G361, C8161, GAK, and MMAc with simvastatin in various concentrations for 1 to 3 days. To investigate the antitumor effect of simvastatin, we analyzed cell viability, morphologic changes, reversibility of inhibition by geranylgeranyl pyrophosphate and farnesyl pyrophosphate, apoptosis and the cell cycle. Simvastatin treatment reduced cell viability in all five melanoma cell lines. The different melanoma cell lines, however, displayed different sensitivities to simvastatin. The addition of geranylgeranyl pyrophosphate to A375M and G361 cells in the presence of simvastatin completely restored the viability of cells, but the addition of farnesyl pyrophosphate did not. DNA fragmentation assay showed that simvastatin induced apoptosis in A375M and G361 cells. Simvastatin caused a G1 arrest in G361 and MMAc cells. Consistent with the cell cycle arrest, simvastatin caused an increase in the mRNA levels of p21 and p27 on G361 and MMAc cells.

We conclude that simvastatin has an antitumor effect on human melanoma cells *in vitro* via apoptosis and cell cycle arrest. These results suggest that simvastatin may be an effective anticancer drug for malignant melanoma. ***Melanoma Res*** **00:000–000 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Melanoma Research 2008, 00:000–000

Keywords: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, apoptosis, cell cycle arrest, G1 arrest, malignant melanoma, melanoma, simvastatin, statins

Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Hokkaido University, Sapporo, Japan

Correspondence to Professor Yuhei Yamamoto, MD, PhD, Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan
Tel: +81 11 706 6978; fax: +81 11 706 7827; e-mail: yu-h1@med.hokudai.ac.jp

Received 18 September 2007 Accepted 18 December 2007

Introduction

Melanoma is a tumor with increasing incidence. Advanced disease is associated with a poor prognosis and responds very poorly to available treatment modalities including chemotherapy and immunotherapy [1,2]. In the early stages, however, melanoma is often curable by surgical excision. Patients with minimally invasive lesions have a 10-year survival rate of greater than 95% [3,4]. In contrast, once the melanoma has spread to the lymph nodes, only 40–50% of these patients survive for 5 years. Moreover, the 5-year survival rate for patients with distant metastases is approximately 10–15% [5]. This emphasizes the need to develop newer approaches to treating advanced melanoma.

AQ1

The statin-class of drugs (i.e. lovastatin, simvastatin, atorvastatin, fluvastatin, pravastatin, cerivastatin, and rosuvastatin) was first marketed in 1987 and is now widely used for the treatment of hypercholesterolemia and for the prevention of cardiovascular disease in high-risk patients [6]. Statins inhibit 3-hydroxy-3-metylglutaryl coenzyme A (HMG-CoA) reductase, the main regulatory enzyme of cholesterol biosynthesis [7]. The safety profile of statins that have been in clinical use for almost two decades is favorable [8,9].

Statins have also been investigated as anticancer agents in various cell lines, including melanoma cell lines, and it has been reported that some of the statins had antiproliferative effect on melanoma cell lines. In a study using murine melanoma models, lovastatin was shown to potentiate antitumor activity of doxorubicin via an apoptosis-dependent mechanism [10]. In human melanoma cell lines, lovastatin has been demonstrated to inhibit cell proliferation to induce apoptosis [11].

In a coculture system using human dermal fibroblasts and human umbilical vein endothelial cells, lovastatin has been shown to reduce angiogenic activity of human melanoma cell lines [12]. Atorvastatin has been reported to inhibit the invasive potential of human melanoma cell lines by preventing rho geranylation [13].

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In cancer research, lovastatin is one of the most frequently studied statins [10–12,14–17], however, lovastatin has not been approved for use yet in Japan. In addition, lovastatin is currently replaced by novel, more potent statins [18]. Therefore, we set out to characterize the response of melanoma cell lines to simvastatin and the effects of the drug on several pathways that are essential for the survival of malignant melanoma cells *in vitro*. Normal fibroblast (NF) cells were used as normal cells, for comparison purpose.

Materials and methods

Simvastatin and cholesterol intermediates

Simvastatin was purchased from Calbiochem (Darmstadt, Germany). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate were purchased from SIGMA (St Louis, Missouri, USA).

Cell cultures

Human melanoma A375M cells were kindly provided by Dr Saiki (Research Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan); GAK cells were obtained from Institute for Fermentation (Osaka, Japan); MMAc, G361, and C8161 were obtained from Riken Cell Bank (Tsukuba, Japan). NF cells were obtained from the normal skin portion of a patient with keloid. Dulbecco's modified Eagle's medium (DMEM) and DMEM/HAM's F12 (DMEM/F12) were purchased from SIGMA (St Louis, Missouri, USA).

A375M, G361, GAK, MMAc, and C8161 were maintained in DMEM/F12 supplemented 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. NF was maintained in DMEM, supplemented with 10% FBS and antibiotics at 37°C in humidified atmosphere of 95% O_2 and 5% CO_2.

Cell proliferation assay

The antiproliferative effect of simvastatin was determined by WST-8 assay using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). WST-8 assay measures the metabolic activity of viable cells, based on the cleavage of the tetrasolium salt, WST-8 by mitochondrial dehydrogenases in viable cells.

Melanoma cells and NF cells were seeded at an initial cell concentration of 5×10^3 cells in 100 µl of medium per well of 96-well culture plates. Simvastatin at various concentrations were added to the wells. After incubation for 24–72 h in DMEM/F-12 without FBS, 100 µl of WST-8 was added to each well and allowed to incubate for 4 h. Assessment of viability was performed according to the manufacturer's instructions. Absorbency was measured by a microplate reader (Emax, Molecular Devices, Sunnyvale, California, USA) with a test wavelength of 450 nm and reference wavelength of 630 nm. Simvastatin was dissolved in dimethyl sulfoxide (DMSO) when used for cell culture studies, and DMSO-treated cells served as normal control. The concentrations of simvastatin tested were 0.5, 1, 2.5, 5, 10, and 15 µmol/l. Relative percentage of cell viability was estimated as mean OD450/630 in test/control × 100. Each determination was performed in triplicate.

Microscopy

A phase-contrast microscope (IX70, Olympus, Tokyo, Japan) equipped with a digital camera (DP11, Olympus, Tokyo, Japan) was used for qualitative morphologic assessment of cells.

Reversibility of inhibition

The effect of adding FPP and GGPP was studied by WST-8 assay in A375M, G361, and MMAc cells to determine whether the influence of simvastatin was reversible. Melanoma cells were seeded at an initial cell concentration of 5×10^3 cells in 100 µl of medium per well of 96-well culture plates. Simvastatin at various concentrations were added to the wells and coincubated with 10 µmol/l FPP or 10 µmol/l GGPP for 72 h in DMEM/F-12 without FBS. DMSO-treated cells served as normal control. The concentration of simvastatin tested were 1, 5, 10 µmol/l. Relative percentage of cell viability was estimated as mean OD450/630 in test/control × 100. Each determination was performed in triplicate.

Detection of DNA fragmentation

Apoptosis is best characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180–200 bp and multiples thereof that are readily detected as a DNA ladder by gel electrophoresis. A375M, G361, and MMAc cells were treated with simvastatin at indicated concentrations for 48 h in DMEM/F-12 supplemented with 0.5% FBS. Genomic DNA was isolated from cells to perform DNA laddering. Briefly, cells were collected, washed in phosphate-buffered saline, and pelleted in an eppendorf tube using 200*g* for 5 min. The supernatant was discarded. Two hundred microliter of lysis buffer (10 mmol/l Tris-HCl buffer, pH 7.4, 10 mmol/l ethylene diamine tetra acetic acid, pH 8.0 and 0.5% Triton X-100) were added, vortexed gently, and placed at 4°C for 15 min. After centrifugation for 10 min at 10 000*g*, the supernatant was aspirated and transferred to new microtubes. Two microliter ribonuclease A (stock solution 10 mg/ml, Wako, Osaka, Japan) was added to each sample and incubated for 1 h at 37°C. Two microliter proteinase K (stock solution 10 mg/ml, Wako, Osaka, Japan) was added to each sample and incubated for 30 min at 50°C. DNA was precipitated by adding 5 mol/l NaCl and isopropanol and stocked at −20°C overnight. After centrifugation for 15 min at 10 000*g*, the DNA pellet was air-dried, resuspended in TE buffer (1 mol/l Tris-HCl buffer, pH

7.4, 0.5 mmol/l ethylene diamine tetra acetic acid, pH 8.0), and its concentration was determined spectrophotometrically. DNA was loaded onto a 1.5% agarose gel in Tris-acetate buffer, and the fragments were electrophoresed at 100 V for 1 h and visualized by staining with ethidium bromide and photographed under ultraviolet light.

Cell cycle analysis

A375M, G361, and MMAc cells were grown in medium of 1% serum conditions on 100-mm plates. At 50% confluency, cells were treated with 0.5, 1, 2 μmol/l simvastatin for A375M cells, 4, 8, 12 μmol/l simvastatin for G361 cells and 1, 2.5, 5 μmol/l simvastatin for MMAc cells, respectively. The above concentrations of simvastatin for each cell line were the most suitable concentration. DMSO-treated cells served as normal control. After 48 h, cells were collected and processed for cell cycle analysis. Briefly, melanoma cells were harvested from monolayer cultures by low-speed centrifugation after incubation for 48 h with simvastatin. Cell pellets were fixed in 1-ml 70% ethanol at −20°C for 30 min. Subsequently, cells were centrifuged at 200*g* for 5 min, then the supernatant was discarded and washed twice in 2 ml of phosphate-buffered saline. One microliter ribonuclease A (stock solution 10 mg/ml, Wako, Osaka, Japan) was added and incubated at room temperature for 5 min. After gentle mixing, 20 μg of propidium iodide (Molecular Probes Inc, Eugene, Oregon, USA) was added. The suspension was incubated in the dark at room temperature for 30 min and then held at 4°C in the dark for flow cytometry. Cell cycle distribution was then analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, California, USA). Finally, the percentages of cells in different phases of cell cycle were determined by ModFit cell cycle analysis software.

AQ2

Reverse transcription polymerase chain reaction

We examined the pattern of expression of key cell cycle regulators [cyclins, cycline dependent kinases (CDKs), cycline-dependent kinase inhibitor (CKIs)] in melanoma cells after treatment with simvastatin. Total cellular RNA was extracted from monolayer cultures of G361 and MMAc cells with TRIzol (Invitrogen, Carlsbad, California, USA). For reverse transcription (RT) (PCR), 3 μg of total RNA was subjected to cDNA synthesis in 100 μl of reaction mixture containing First-Strand Buffer (Invitrogen, Carlsbad, California, USA), 200 U/μl of Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, California, USA), 1-μg Random Primers (Invitrogen), 5 mmol/l dithiothreitol (Invitrogen), 0.25 mmol/l dNTP Mix (Applied Biosystems, Foster City, California, USA). The RT reaction was performed sequentially for 2 h at 37°C and for 10 min at 70°C. PCR amplification of cDNA was performed in 20 μl of reaction mixture containing 1 μl of cDNA sample, 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 1.25 U/μl AmpliTaq DNA Polymerase, and 1.5 pmol of primers. Cycling conditions in terms of the number of cycles and annealing temperature were optimized for each pair of primers (Table 1) [19–28]. cDNA of the *GAPDH* gene was used as an internal control. PCR conditions were as follows: at 95°C for 10 min, 28–32 cycles; at 94°C for 1 min, for 40 s at appropriate annealing temperatures, 40 s at 72°C, and at the end of the amplification at 72°C for 10 min. PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

Data are expressed as mean values and the standard error of the mean. Statistical analysis was performed using two-tailed unpaired *t*-test when comparing two groups. $P < 0.05$ was taken to indicate the significance.

Results

Simvastatin inhibited cell growth of human melanoma cells

We examined the growth inhibitory effects of simvastatin from five melanoma cell lines and normal skin fibroblast

Table 1 Primers for real time-PCR analysis

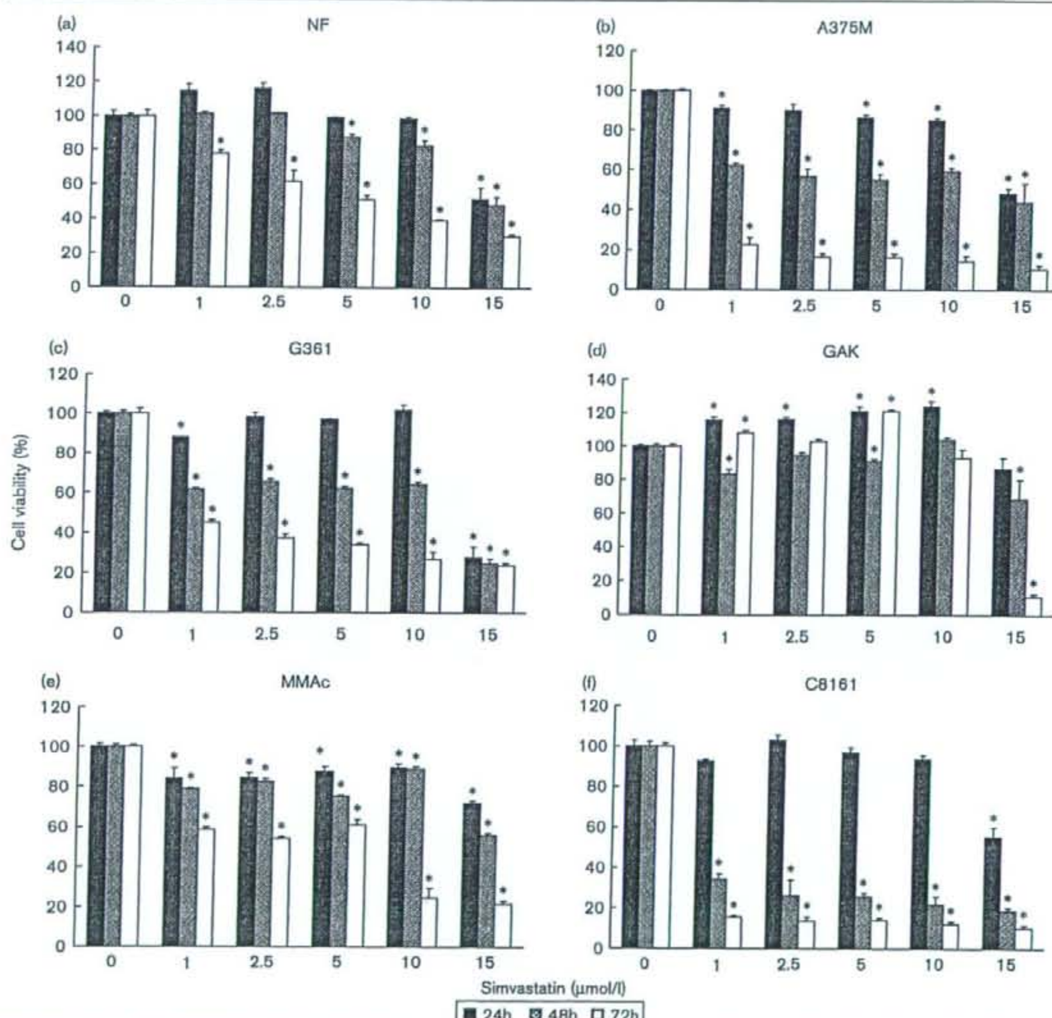
AQ4

Gene	Primer type	Primer sequence	Size	Tm (°C)	Cycle number	Reference
CDK2	Sense	GCTTTCTGCCATTCTCATCG	217	57	28	[19]
	Antisense	GTCCCCAGAGTCCGAAAGAT				
CDK4	Sense	ACGGGTGTAAGTGCCATCTG	488	60	28	[19]
	Antisense	TGGTGTCGGTGCCTATGGGA				
CDK6	Sense	CGAATGCGTGGCGGAGATC	499	62	32	[19]
	Antisense	CCACTGAGGTTAGAGCCATC				
cyclinD1	Sense	ACCTGGATGCTGGAGGTCTG	405	63	28	[20]
	Antisense	GAACTTCAGATCTGTGGCACA				
cyclinE	Sense	ATACAGACCCACAGAGACAG	301	62	32	[19]
	Antisense	TGCCATCCACAGAAATACTT				
p21	Sense	GCGCTAATGGCGGGCTGCAT	366	55	29	[21]
	Antisense	GCCGGCGTTTGGAGTGGTAG				
p27	Sense	ATGTCAAACGTGCGAGTGTC	250	59	28	[22]
	Antisense	TCTGTAGTAGAACTCGGGCAA				
GAPDH	Sense	TGAAGGTCGGAGTCAACGGATTTG	983	59	28	[23]
	Antisense	CATGTGGGCCATGAGGTCCACCAC				

cell line, using WST-8 assay (Fig. 1). Simvastatin treatment reduced cell viability of melanoma cells in a time-dependent manner except GAK cells. Cell viability was over 80% at concentrations ranging between 1 and 10 μmol/l in 24 h of incubation, but at 15 μmol/l G361 cells displayed more than 70% decrease in viability and for A375M and C8161 cells approximately 50% decrease in viability. MMAc cells displayed approximately 30% decrease in viability, and GAK cells displayed approximately 10% decrease in viability.

Cell viability was not decreased in a dose-dependent manner at concentrations ranging between 1 and 10 μmol/l in 48 h of incubation with simvastatin except C8161 cells. All melanoma cell lines except GAK cells, regardless of their variance, displayed a decrease in viability in a dose-dependent manner at 72 h. As shown in Fig. 1, WST-8 assay showed different toxicity profile. Simvastatin inhibited cell growth of A375M and C8161 cells strongly, but as for GAK, most weakly.

Fig. 1



Proliferation assay. Growth inhibitory effects of simvastatin on various melanoma cell lines and normal fibroblast (NF) cells. NF (a), A375M (b), G361 (c), GAK (d), MMAc (e), and C8161 (f) cells were plated and treated with 0.5, 1, 2, 5, 5, 10, or 15 μmol/l for 24, 48, and 72 h. Viable cells were determined by WST-8 assay read at 450 nm with reference wavelength of 630 nm. Results are expressed as percentage of cell growth compared with that untreated controls and are mean ± SEM of triplicate determinations. * $P<0.05$ compared with control.

Morphologic change of melanoma cells

As statins cause cell rounding by preventing isoprenylation of proteins important for cytoskeletal organization [19,20], we studied the effect of simvastain on the morphology of melanoma cells (Fig. 2). Examination of the effect of exposure to simvastatin on A375M, G361, and MMAc cells by the phase-contrast microscopy revealed that the cells lost their adherent phenotype and assumed a circular morphology. Untreated melanoma cells were adherent to the dish and were spindle shaped. A375M cells were rounded and detached beginning from a concentration of 1 μmol/l. G361 cells were detached beginning from a concentration of 5 μmol/l, and the number of rounded cells and detatched cells increased at 10 μmol/l. MMAc cells were detached beginning from a concentration of 1 μmol/l. Adherent cells were observed, but the number of rounded cells was increased at a concentration of 10 μmol/l.

Reversal of simvastatin-induced inhibition of cell growth in melanoma cells by geranylgeranyl pyrophosphate

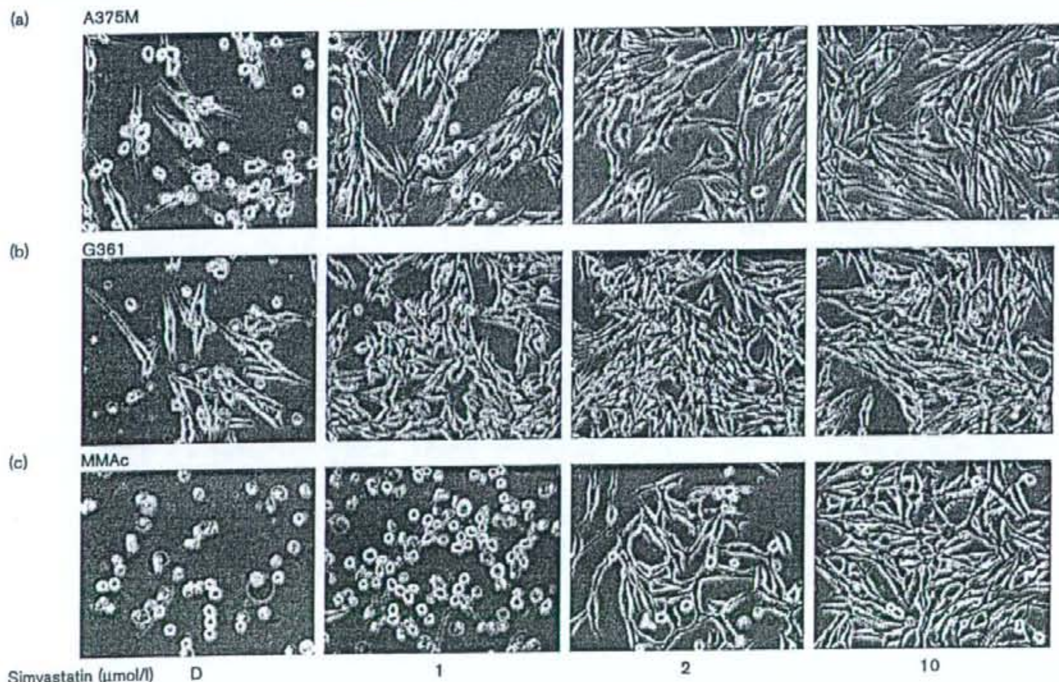
Prenylation of proteins by FPP or GGPP is essential for small G-protein function, which include cell growth, cell differentiation, and cell survival (Fig. 3) [20–22]. We assessed the involvement of mevalonate metabolites in simvastatin-treated melanoma cells by addition of FPP and GGPP into the culture medium (Fig. 4).

GGPP completely abolished the simvastatin-induced effects, but FPP did not with A375M and G361 cells. With MMAc cells, GGPP was able to restore cell viability, but the effect was smaller than with A375M and G361. These data indicated that the inhibition of geranylgeranylation of small G-protein is implicated in the decreased cell viability of melanoma cells.

Simvastatin induced apoptosis of A375M and G361 cells

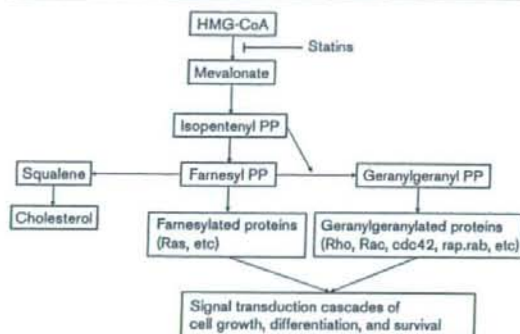
One of the hallmarks of apoptosis is the endonuclease-mediated degradation of chromatin, giving rise to characteristic DNA laddering [23]. To investigate the effect of the simvastatin on DNA fragmentation, A375M, G361, and MMAc cells were incubated with simvastatin at the indicated concentrations for 72 h, and then the DNA was extracted and separated by agarose gel electrophoresis.

Fig. 2



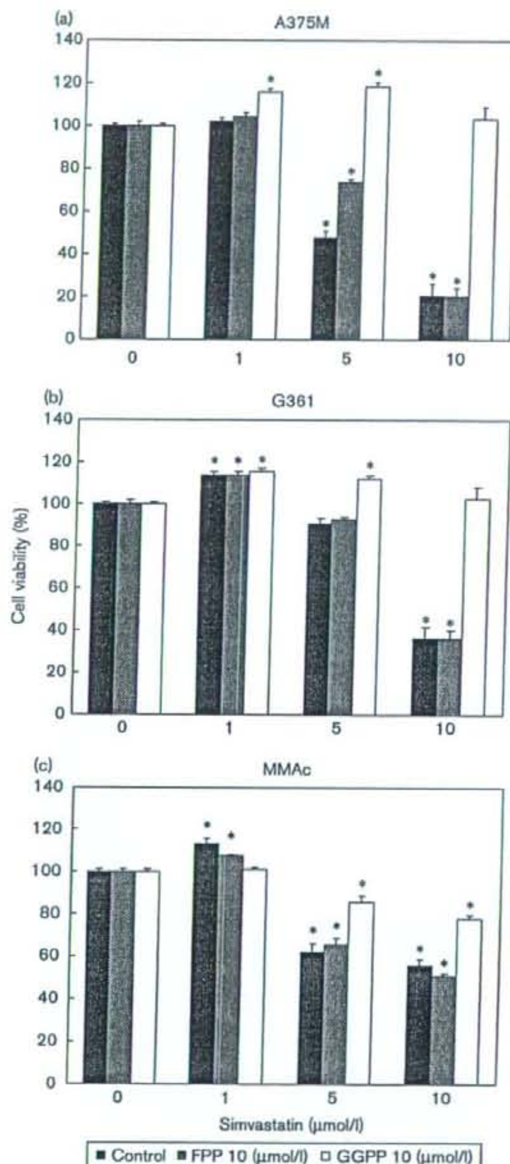
Morphologic change. Morphology of A375M (a), G361 (b), and MMAc (c) cells treated with simvastatin at 1, 5, or 10 μmol/l for 72 h. Cells were observed using phase-contrast microscopy. (magnification × 100).

Fig. 3



Mevalonate cascade.

Fig. 4



Add-back experiment. Reversibility of simvastatin-induced inhibition of cell growth in melanoma cells by intermediates of the mevalonate pathway: farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). A375M (a), G361 (b), and MMAc (c) cells were co-cultured with simvastatin at indicated concentrations and 10 µmol/l of FPP or 10 µmol/l of GGPP for 72 h. Viable cells were determined by WST-8 assay read at 450 nm with reference wavelength of 630 nm. Results are expressed as percentage of cell growth compared with that untreated controls and are mean ± SEM of triplicate determinations. *$P<0.05$ compared with control.

The pattern of DNA fragmentation was apparent when A375M cells were treated with 5 µmol/l simvastatin and G361 with 20 µmol/l simvastatin, but was not apparent when simvastatin was used for treating MMAc cells (Fig. 5). This indicated that simvastatin induced apoptosis in A375M cells and G361 cells.

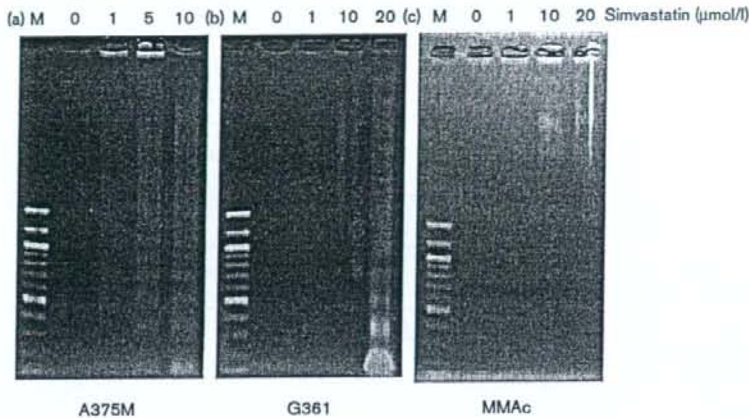
Simvastatin caused cell cycle arrest of G361 and MMAc cells

As we observed a growth inhibitory effect of simvastatin, we then analyzed its possible effect on cell cycle progression after treatment with various concentrations of simvastatin for 48 h. The percentage of cells in each cell cycle phase (G1, S, and G2/M) was determined by flow cytometry (Fig. 6). G361 and MMAc cells showed an increase in the relative percentage of cells in G1 phase and corresponding decrease in the number of cells in S phase. No significant effect on cell cycle progression was observed in A375M cells. These results indicated that simvastatin induced G1 arrest in G361 and MMAc cells.

Simvastatin induced increase in the mRNA levels of p21 and p27 on G361 and MMAc cells

In an attempt to probe the molecular mechanism involved in the G1 arrest induced by simvastatin, we investigated key cell cycle regulatory gene in G361 and MMAc cells. Cells treated with simvastatin (10 µmol/l) were incubated for 48 h, the total RNA was extracted and the expression of CDKs (CDK2, CDK4, CDK6), cyclins (cyclin D1, cyclin E), and CKIs (p21, p27) was studied by RT-PCR analysis. When compared with control, simvastatin treatment resulted in an increase in the expression of p21 and p27 with G361 and MMAc cells (Fig. 7). In this study, simvastatin treatment did not show any alteration in mRNA levels of CDK2, CDK4, CDK6, cyclin E, and cyclin D1. These results indicated that an increase in CKIs after simvastatin treatment.

Fig. 5



DNA fragmentation assay. DNA fragmentation induced by simvastatin. A375M (a), G361 (b), and MMAc (c) cells treated with simvastatin at indicated concentrations for 48 h were lysed in lysis buffer, and genomic DNA extracts obtained were electrophoresed on a 1.5% agarose gel. The result is a representative of three individual experiments.

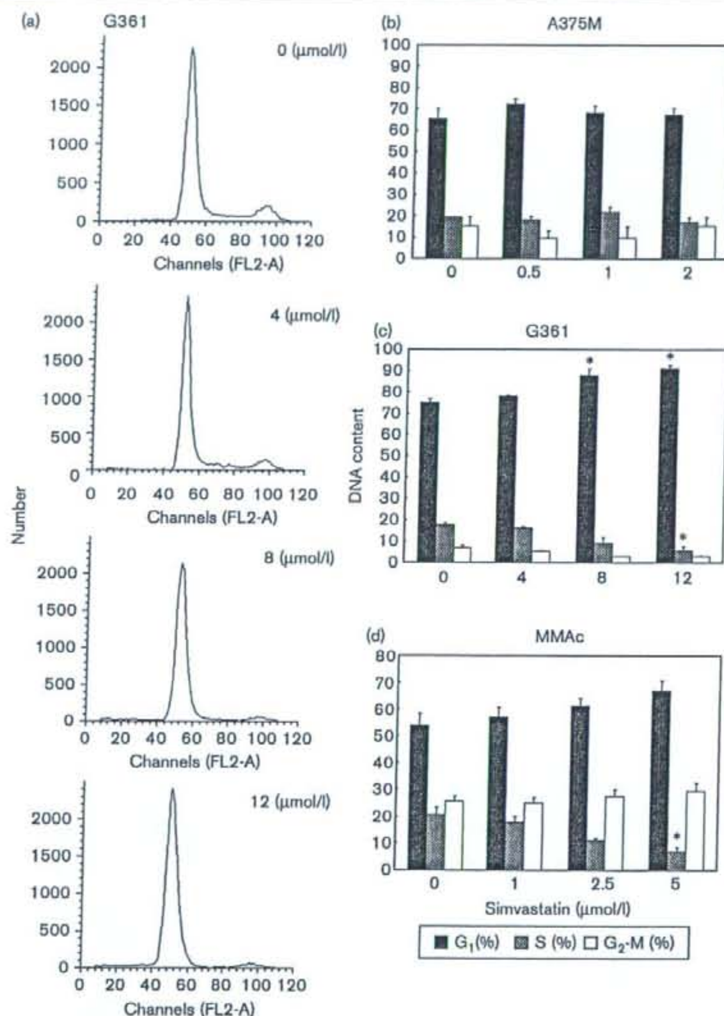
Discussion

Statins are inhibitors of HMG-CoA reductase and is known to block the mevalonate pathway. Recently, Clinical trials, investigating the possible value of statins as anticancer agents have been conducted. The reports on the relationship between statin use and melanoma incidence have yielded mixed results, with no associations [29], as well as with positive associations being observed [30,31]. In contrast, statins have been demonstrated to exert antitumor effects on melanoma cells in-vitro and in-vivo studies. The molecular mechanisms proposed to explain the anticancer properties of statins on melanoma cell lines include induced-apoptosis [11] and inhibition of rho geranylation [13]. The effect of cell cycle arrest, however, has not yet been suggested. Furthermore, among the statin class of drugs, the antitumor effects of simvastatin on human melanoma cells have not been investigated in depth yet. In this study, we showed that simvastatin is effective in inhibiting the growth of various melanoma cell lines and also induce apoptosis and a G1 cell cycle arrest via the induction of p21 and p27 as an underlying mechanism in melanoma cells.

This study showed that simvastatin was effective in abrogating melanoma cell grown *in vitro*, however different melanoma cell lines displayed different sensitivities to simvastatin. GGPP and FPP are intermediates of the mevalonate cascade and are used for protein prenylation. This posttranslational modification involves the covalent attachment of GGPP or FPP to regulatory proteins such as Ras and rho and facilitates their association to the plasma membrane where they fulfill their function in cell signal transduction and cytoskeletal organization [32,33]. It has been reported that the geranylgeranylated rho family members play important roles in various cellular processes such as cell differentiation, cell proliferation, and apoptosis [15,22]. Moreover, many studies have demonstrated that GGPP reversed statin inhibition of rho-mediated responses [14,15,22,34]. This study demonstrated that the inhibitory effect of simvastatin was reversible by the addition of GGPP but not with FPP. This finding indicates that the inhibition of the geranylgeranylation of signaling molecule is implicated regarding the effects of simvastatin on melanoma cells.

Apoptosis is an active cell death mechanism that plays a critical role in several biological processes [35]. Many antitumor agents have been reported to induce apoptosis in several tumor cells, and apoptosis is suggested to be one of the major mechanisms for the targeted therapy of melanomas [36]. The cells within melanoma lesions demonstrate an inherently low level of spontaneous apoptosis [37], and resistance to apoptosis has been correlated with increased metastatic potential in animal models of melanoma [38]. Recently, the expression of antiapoptotic and proapoptotic mediators of apoptosis pathways in melanoma was investigated, and it is reported that proapoptotic Bcl-2-related proteins Bax and Bak in melanoma is associated with worse patient prognosis [39,40]. Thus, it is likely that a pharmacological agent acting through apoptotic activation alone, or in combination with conventional cytotoxic drugs, may

Fig. 6

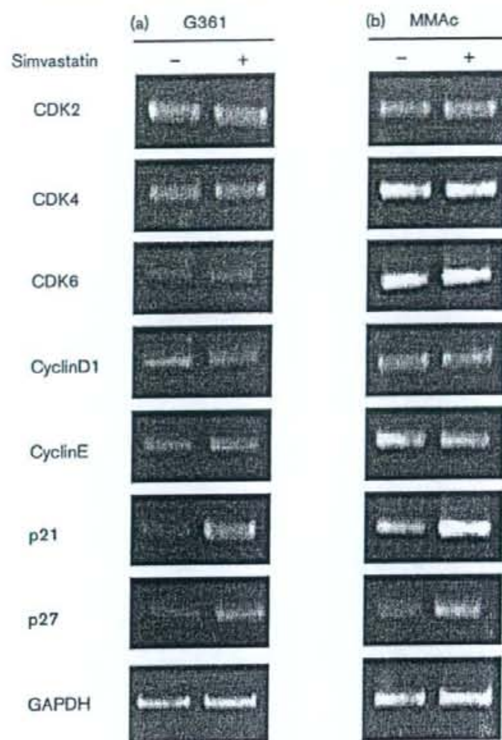


Cell cycle analysis. Effect of simvastatin on cell cycle progression of melanoma cells. Cells treated with simvastatin at indicated concentrations for 48 h were harvested, washed with PBS, and digested with RNase. Cellular DNA was stained with propidium iodide, and flow cytometric analysis was done to determine the cell cycle distribution. (a) Propidium iodide fluorescence pattern for cell cycle distribution in different treatments in G361 cells. (b–d) the percentage of cell cycle distribution data for each treatment group in G361, MMAc, and A375M cells. Results are mean ± SEM of three independent experiments. *$P<0.05$ compared with control.

improve the prognosis for melanoma patients. In this report, we were able to show an induction of apoptosis in A375M cells and G361 cells by simvastatin. Therefore, the agents could be useful in controlling melanoma cells. We, however, did not observe the DNA laddering pattern with MMAc cells. The DNA laddering pattern may be due to the resistance to apoptosis by MMAc cells. In addition, it was, however, necessary to investigate further the mechanism for the differences in the responses to the induction of apoptosis.

As the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies [41,42], control of cell cycle

Fig. 7



Real time (RT)-PCR analyses of various cell cycle regulators 48 h after treatment of melanoma cells G361 and MMAc cells with 10 μmol/l simvastatin (+) or untreated controls (–). GAPDH was used as an internal control. Results are from an experiment representative of three similar experiments.

progression in malignant tumor cells is considered to be a potentially effective strategy for the control of tumor growth [43,44]. Our data elucidated that the treatment of G361 cells and MMAc cells treated with simvastatin resulted in G1 arrest of the cell cycle progression, which indicated that one of the mechanisms by which simvastatin may act to inhibit the proliferation of melanoma cells is the inhibition of cell cycle progression.

CDKs, cyclins, and CKIs play critical roles in the regulations of cell cycle progression [44]. CKIs are tumor suppressor proteins that downregulate the cell cycle progression by binding with active CDK–cyclin complexes and thereby inhibiting their kinase activities [43,44]. p21 is a direct transcriptional target of p53 and strongly induced by wild-type p53 in response to DNA damage. It mediates the growth suppression effects of p53 by arresting the cell cycle at the G1/S checkpoint and by inducing apoptosis [45]. p27 has been demonstrated to play an important role in regulating progression through G1 and entrance into S phase of the cell cycle by binding to and preventing premature activation of cdk4/cyclin D and cdk2/cyclin E complexes [46,47]. It was reported that treatment of cancer cells by statins resulted in the induction of p21 and p27 [48–52]. In this report, cell cycle analysis data showed that simvastatin caused a G1 arrest in cell cycle progression of G361 cells and MMAc cells. Furthermore, mechanistic investigation by RT-PCR suggested that simvastatin-induced G1 arrest in melanoma cells were thought to be mainly mediated via an upregulation of p21 and p27. Recent studies have also indicated that the alterations of CKIs level may have prognostic significance in melanoma [53]. Expression of p21 has been reported to be inversely associated with high AJCC stage and recurrence free survival [54]. It was reported that decreased expression of p27 was significantly associated with increasing tumor thickness and that low expression of p27 was correlated with reduced disease-free survival in primary nodular melanomas [55]. In this report, we showed that simvastatin induced cell cycle arrest and overexpression of p21 and p27. Therefore, they may be effective agents in controlling melanoma cell growth.

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In conclusion, the results of this study demonstrated that simvastatin has an antiproliferative effect *in vitro* against human melanoma cell lines, and this effect was due to, at least in part, the induction of apoptosis and a cell cycle arrest. Although further investigations are needed to elucidate the precise mechanisms by which simvastatin inhibits cell proliferation; currently simvastatin may be considered to be a useful tool for malignant melanoma therapeutic regime.

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