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A link between metastasis and resistance to apoptosis of variant small cell lung carcinoma

Emma Shtivelman

Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, California, 94115 USA

A novel human gene CC3 with properties of a metastasis suppresor gene for small cell lung carcinoma (SCLC) is described. CC3 is an evolutionary conserved gene that is expressed ubiquitously in human tissues. CC3 RNA is absent in a subset of SCLC cell lines known as variant (v-SCLC) that are derived from tumors characterized by highly aggressive metastatic behavior. Introduction of CC3 into a variant SCLC line results in significant suppression of its metastasis *in vivo*. When deprived of growth factors *in vitro*, v-SCLC cells modified to express CC3 undergo rapid and massive cell death that at least partially could be ascribed to the activation of the apoptotic pathway. In addition, expression of CC3 in v-SCLC cells increases induction of apoptosis by chemoterapeutic drugs. Loss of CC3 in highly metastatic cells such as SCLC might render them resistant to death-inducing signals and thus help to ensure their survival under unfavorable conditions encountered in the metastatic process.

Keywords: metastasis; apoptosis; SCLC

Introduction

One quarter of all lung cancer cases are diagnosed as SCLC, which has the poorest survival rate of all histologic types of lung cancer. The pathology, etiology, clinical course and biology of SCLC are different from other forms of lung cancer (Carney and De Leij, 1988). SCLC is the most radio- and chemosensitive among all the histologic types of lung cancer. However, though the initial response rate of SCLC to cytotoxic therapy is high, the vast majority of patients will experience a relapse of the disease, so that only less than 5% of all patients will be cured (Sieffer and Ihde, 1988). The hallmark feature of SCLC is its propensity for aggressive metastatic dissemination: approximately 70% of patients will have disease involving at least one extrathoracic site already at the time of presentation (Dearing *et al*., 1990).

SCLC displays a number of neuroendocrine characteristics that are shared with the normal neuroendocrine cells of lung (Carney *et al*., 1985; Gazdar *et al*., 1988; Weidenmann *et al*., 1986; Jensen *et al*., 1990; Bunn *et al*., 1985). In contrast to the classic SCLC (c-SCLC) subset that express an array of neuroendocrine markers and comprise about 70% of SCLC, a separate subtype of variant SCLC (v-SCLC) is recognized that is characterized by the partial loss of the neuroendocrine phenotype (Carney *et al*., 1985; Gazdar *et al*., 1985). The v-SCLC cell lines are also distinguished by rapid growth *in vitro* and altered morphological characteristics. Clinically tumors of v-SCLC subtype have an even poorer prognosis than c-SCLC and are frequently resistant to standard chemo- and radiotherapy (Radice *et al*., 1982; Carney *et al*., 1983; Morstyn *et al*., 1984; Aisner *et al*., 1990).

The molecular mechanisms that are responsible for the highly metastatic phenotype of SCLC are poorly understood. This is due mostly to the fact that the metastatic character of SCLC is difficult to reproduce in immunodeficient rodents. However, SCLC cells are metastatic in a species-specific manner in SCID-hu mice, i.e. severe combined immunodeficiency mice engrafted with fragments of human fetal tissues (Shtivelman and Namikawa, 1995). SCLC cells injected intravenously into SCID-hu-L mice carrying grafts of human fetal lung (HFL) produce metastatic tumors in HFL grafts but not in mouse lungs or other mouse tissues (Shtivelman and Namikawa, 1995). v-SCLC lines are significantly more metastatic in SCID-hu-L mice than c-SCLC: intravenous injection of as few as 10^5 cells results in tumors in HFL grafts of all animals, while c-SCLC lines produce metastatic tumors in HFL grafts of only a fraction of experimental animals even when millions of cells are injected (Shtivelman and Namikawa, 1995).

Molecular analysis of gene expression was performed to find genes that might be responsible for the highly metastatic phenotype of v-SCLC. The novel gene named CC3 was identified on the basis of its preferential expression in weakly metastatic c-SCLC lines but not in highly metastatic v-SCLC. Its inhibitory effect on metastasis *in vivo* described below is consistent with the hypothesis that CC3 is a metastasis-suppressor gene for variant SCLC. This effect might be at least in part mediated by the ability of CC3 to promote apoptotic death of v-SCLC cells.

Results

Identification of CC3

Differential display of RNA (Liang and Pardee, 1992) was used to compare mRNA populations of v-SCLC lines NCI-N417 and NCI-H82 that are highly metastatic to HFL grafts *in vivo* and the weakly metastatic c-SCLC lines NCI-H146, NI-H345, and NCI-H69 (Shtivelman and Namikawa, 1995). A cDNA fragment designated CC3 was identified that was preferentially expressed in the three c-SCLC lines but not in v-SCLC N417 or H82 (Figure 1a). Sequence of this cDNA was found to be identical to several

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human cDNA clones found in the databases of expressed sequence tags, but no similarities to other human genes were detected.

Expression of CC3 was examined in normal tissues (Figure 1b) and a number of human tumor cell lines (some of which are shown in Figure 1c). A 1.6 kb RNA transcript was identified in all normal tissues and tumor cell lines examined exclusive of v-SCLC and neuroblastoma. Various other tumor cell lines showed great variability in the levels of CC3 RNA (data not shown). A longer cDNA clone for CC3 was obtained from a cDNA library constructed with RNA of H146 cells. The most 5′ end sequences of CC3 RNA were retrieved using the RACE technique (Frohman *et al*., 1988). The combined length of cDNA clones was 1.6 kBp, matching the predicted size of CC3 RNA. Sequencing of CC3 cDNA clones revealed an open reading frame coding for a polypeptide of 242 amino acids starting with a first methionine codon at position 99 from the 5′ end. Searches of the sequence databases produced significant homology scores with products of predicted protein coding regions from genomes of three species: *C elegans* (CELC33F10), *S cerevisiae* (SCE9537) and *E Coli* (ECOUW67) indicating a strong evolutionary conservation (Figure 2). All three predicted polypeptides are products of the genome sequencing projects and as such have no assigned function. Additional database searches did not reveal significant homologies between the predicted amino acid sequence of CC3 and any known protein domains.

CCS suppresses metastasis of v-SCLC in SCID-hu-L mice

To examine whether CC3 expression in v-SCLC cells can suppress their metastasis *in vivo*, CC3 cDNA was subcloned into constitutive expression vector pcDNA3 (InVitrogen). The resulting construct pcCC was electroporated into N417 and into a selected clonal population of N417, N417 cl.1. Following selection in G418 containing medium a total of seven single cell clones containing the pcCC construct were successfully propagated. Four clones derived from the original cell line N417 (N417cc1, N417cc2, N417cc5 and N417cc6) and two clones derived from N417 cl.1 (N417cc11 and N417cc12) were examined for expression of CC3 RNAs (Figure 3). Clones N417cc2 and N417cc11 had CC3 RNA levels similar to these in a weakly metastatic c-SCLC line H146 (Figure 3). The other four clones had lower levels of CC3 RNA, and clone N417cc6 had no detectable CC3 RNA.

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CC3          MAETEALSKLREDFRMQNKSVFILGASGETGRVLLKEILEQGLFSKVTLIGRRKLT
CELC33F10                    MSSAFVVGATGAVGSELVKLLAESTKFSKVVVLARRPVD
SCE9537                       MNGLVLGATGLCGGGFLRHAQEAPQFSKVYAILRRELP
ECOUW67        MSRGAQGRQFCKGAKMSQVLITGATGLVGGHLLRMLINEPKVNAIAAPTRRPLG

FDEEAYKNVNQEVVDFEKLDDYASAFQGH  DVGFCCLGTTRGKAGA EGFVRVDRDYVLKSAELA
GATGD  KLI  QKTVDFDKLEENAEDIQGV  DVAFCALGTTRGKSGA DGFYKVDHDYVMSAAKMA
FPATD  KVVAIVERDNSKWSQLITNEMNP  QVLFTALATTRAAAGGLDKQYKIDHDLNLQLAQAA
       DMPGVFNPHDPQLSDALAQVTDPIDIVFCCLGTTRREAGSKEAFIHADYTLVVDTALTG

KAGGCKHFNLLSSKGADKSSNFLYLQVKGEVEAKVEELKFDRYSVFRPGVLLCDRQESRP   GE
KENGVKQFVLVSSVGADASSRFLYPKTKGEVEKEIGELNFEKFVIMRPGLIEAKRPEFRI   GE
KEKGCETIVLVSSAGAHPDSRFGYMKMKGEIERDVIALDFKHIIILRPGPLLGERTNSKQSGFGG
RRLGAQHMLVVSAMGANAHSPFFYNRVKGEMEEALIAQNWPKLTIARPSMLLGDRSKQRM   NE

WLVRKL  FGSLPDSWAR   GHSVPVVTVVRAMLNNVVRPRDKQ MELLENKAIHDLGKAHGSLKP
FLGKIV  TAPLGLFSNR    FSSSATAIAQAMINATQTEETGN  QIWNNSKIVEESKKYTA
NLTAALGTRVYRSRFQRLLGYPVYGDEVGKVGVHLALNTSGKDKVQFVSSKDILDISASLEKIAT
TLFAPL  FRLLPGNWKS      IDARDVARVMLAESMRPEHEG  VTILSSSELRKRAE
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Figure 2 Predicted amino acid sequence of CC3 and alignment with homologous sequences. Amino acid residues that are identical or conserved in at least three out of four predicted proteins are shown in black. Conservative replacements are grouped as I, L, M, V; F, W, Y; D, E, N, Q; H, K, R; A, G, P, S, T. The degrees of amino acid identity and conservation are respectively 41 and 62% between CC3 and the *C elegans* predicted gene product CELC33F10, 31 and 47% between CC3 and the *S cerevisiae* gene SCE9537 and 27 and 43% between CC3 and the *E coli* gene ECOUW67. The sequence of CC3 was deposited in the GenBank under accession number U69161

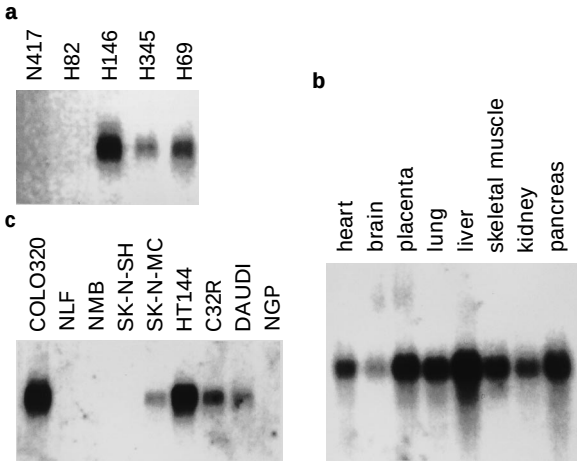

Figure 1 Northern blot analysis of CC3 mRNA expression. 2 μg of polyadenylated RNA were electrophoresed, blotted and hybridized to a ^{32}P-labeled CC3 cDNA probe. (**a**) RNA from v-SCLC (1,2) and c-SCLC lines (3–5); (**b**) normal human tissues (Clontech blot); (**c**) human tumor cell lines established from neuroblastomas (NGP, NLF, NMB, SKNSH), colon carcinoma (COLO320DM), neuroepithelioma (SK-N-MC), melanomas (HT144 and C32R) and Burkitt lymphoma (Daudi)

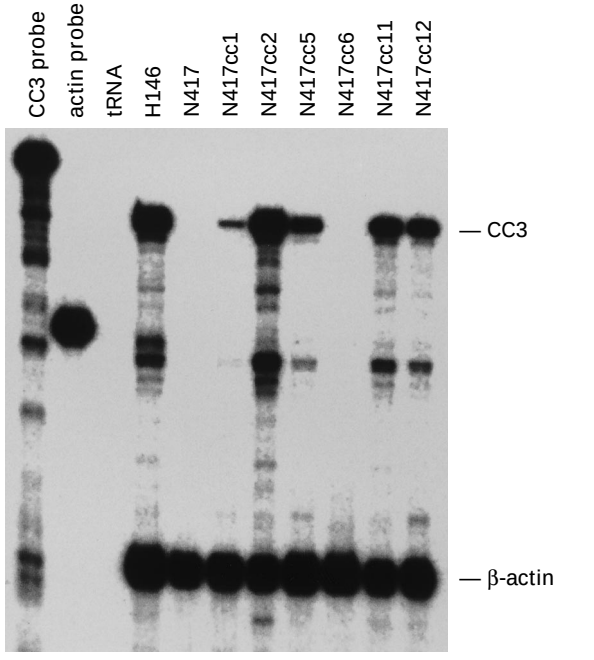

Figure 3 Analysis of CC3 RNA expression in N417 clones. 5 μg of total RNA were hybridized with ^{32}P-labeled antisense RNA probes for CC3 and β-actin. After RNase digestion of single-stranded RNAs the protected products were resolved in denaturing polyacrylamide gel and autoradiographed. Protected fragments specific for CC3 and for β-actin are respectively 280 and 120 nucleotides long. The specific radio-activity of the β-actin probe was adjusted to be about fivefold lower than that of CC3 probe

An expression plasmid pcCCRE was constructed that was identical to pcCC except that CC3 cDNA was subcloned in reverse orientation. pcCCRE was introduced into N417 cells as a control. G418 resistant single cell clones harboring the control construct were selected in the same manner as experimental clones.

Clones N417cc1, 2, 11 and 12 were injected intravenously into SCID-hu-L mice in the *in vivo* experimental metastasis assay. The following clones were injected as controls: N417cl.1 and N417cl.3 selected from N417, three clones containing the control pcCCRE construct (N417ccre13, Nr17ccre7, N417ccre8) and clone N417cc6 that had no detectable CC3 RNA (Figure 3). The number of cells injected per mouse was chosen as one hundred thousand for the following reason: intravenous injection of 10^5 cells of v-SCLC cell lines N417 and H82 results in metastatic tumors in 100% of experimental animals, while injection of the same number of c-SCLC cells H146 and H345 does not produce tumors (Shtivelman and Namikawa, 1995 and unpublished results).

As expected, control clones have induced metastatic tumors in the HFL grafts of almost all experimental animals at 6 weeks after injection (Table 1). However, the metastatic activity of the CC3-expressing clones was severely impaired: only two out of 30 SCID-hu-L mice that received N417 cells expressing CC3 have developed tumors in HFL grafts at 8 weeks after injection. One of these tumors was a micrometastatic lesion that was discovered only after histologic examination. Intravenous injection of as many as 5×10^5 cells of clones N417cc2 and N417cc12 produced metastatic tumors in only two out of five SCID-hu-L each. This low metastatic potential of CC3 expressing N417 cells is similar to that observed for classic c-SCLC lines (Shtivelman and Namikawa, 1995). Evidently, introduction of CC3 into highly metastatic N417 cells greatly suppresses their ability to form metastatic tumors in HFL grafts. CC3 RNA expression was examined in tumors induced by injection of high cell numbers of N417cc2 and N417cc12 and was found to persist in two out of three tumors analysed (not shown).

The inability of N417 clones expressing CC3 to induce metastatic growth could be at least partly due to an impairment of their ability to grow as tumors *in vivo*. To address this possibility, N417 clones were injected directly into HFL grafts. Twenty-five thousand cells of N417cl.1., N417ccre7, N417cc6, N417cc2, N417cc11 and N417cc12 were injected into HFL grafts of five SCID-hu-L mice each. Mice were euthanized at 5 weeks after injection. With the exception of clone N417cc11, all cells grew as tumors in all injected grafts. The size of tumors induced by N417cc2 and N417cc12 was somewhat smaller than the average for control clones, but no stastistical significance could be assigned to these data due to the small number of experimental animals. N417cc11 cells produced small tumors in only two out of five injected grafts. The impaired ability of clone N417cc11 to form primary tumors might be related to the relatively slow growth rate of this clone *in vitro*. RNA analysis of several primary tumors induced by direct injection of CC3 expressing clones demonstrated presence of the CC3 RNA in these tumors (data not shown). Thus, suppression of the metastatic ability of N417 cells induced by CC3 expression is much more dramatic than its effect on the growth of primary tumors.

Table 1 Metastatic activity of N417 clones in SCID-hu-L after intravenous injection

Cells injected[a]	*Mice with tumors*	*HFLs with tumors*[b]
N417	10/10	15/20
N417 cl.1	9/9	13/18
N417ccre13	5/5	10/10
N417ccre7	8/9	11/18
N417ccre8	5/5	5/10
N417cc6	5/5	6/10
N417cc1	1/5	1/10
N417cc2	1/10[c]	1/20
N417cc11	0/10	0/20
N417cc12	0/5	0/10

[a]SCID-hu-L mice were injected intravenously with 10^5 cells. [b]Each SCID-hu-L mouse had two HFL grafts. [c]Micrometastasis

CC3 expression predisposes v-SCLC cells to apoptosis

The effect of CC3 gene product on the *in vitro* growth characteristics of N417 clones was examined by comparing the growth rates of control and experimental clones and their ability to form colonies in soft agarose. As shown in Table 2, expression of CC3 in N417 cells results in only moderate growth retardation *in vitro*, as well as reduced ability to form colonies in semi-solid agarose. Direct comparison of clones N417cc12 and N417cc11 with their parental clone N417cl.1 shows that their generation time was lengthened by about 30 and 50% respectively, while colony formation is reduced by about two- to 2.5-fold.

Proliferation *in vitro* of CC3 expressing clones differed from that of control cells in that the latter continued to proliferate in undisturbed suspension cultures even when cell concentration exceeded 2×10^6 cells/ml, while the former ceased to accumulate at about 10^6 cells/ml. These 'old' cultures contained a significant percentage of dead cells. The likely reason for this could be the increased sensitivity of CC3 expressing clones to the depletion of growth factors in the medium.

The possibility that CC3 expressing clones undergo cell death as a result of growth factor withdrawal was examined by shifting growth conditions to medium containing only 0.5% serum. Cultures of N417cc2, N417cc11, N417cc12 and control clones N417cl.1., N417cc6 and N417ccre8 were incubated in medium with low serum and analysed for cell viability. As shown in Figure 4 the viability of CC3 expressing

Table 2 Proliferation and agarose colony formation by N417 clones

Clone	*Doubling time, hours*	*No. of colonies per* 5×10^3 *cells*
N417 cl.1	24.5	500 ± 33
N417cc6	28.1	418 ± 28
N417ccre8	23.5	714 ± 44
N417cc2	32.6	315 ± 41
N417cc11	36.6	193 ± 78
N417cc12	32.7	266 ± 35

Doubling times were determined in at least three independent experiments for each clone; the standard error margin did not exceed 5% of the average. Numbers of colonies shown are means of at least two experiments performed in triplicates $\pm$ s.e.m

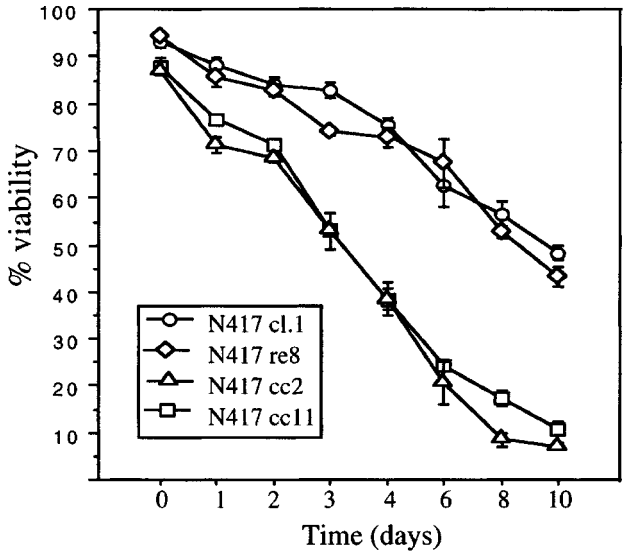

Figure 4 Viability of N417 clones in medium with low serum content. Percentage of living cells in cultures maintained in medium containing 0.5% serum was calculated on the basis of Trypan blue exclusion. Data points represent means of three to five independent experiments. Bars, standard deviations

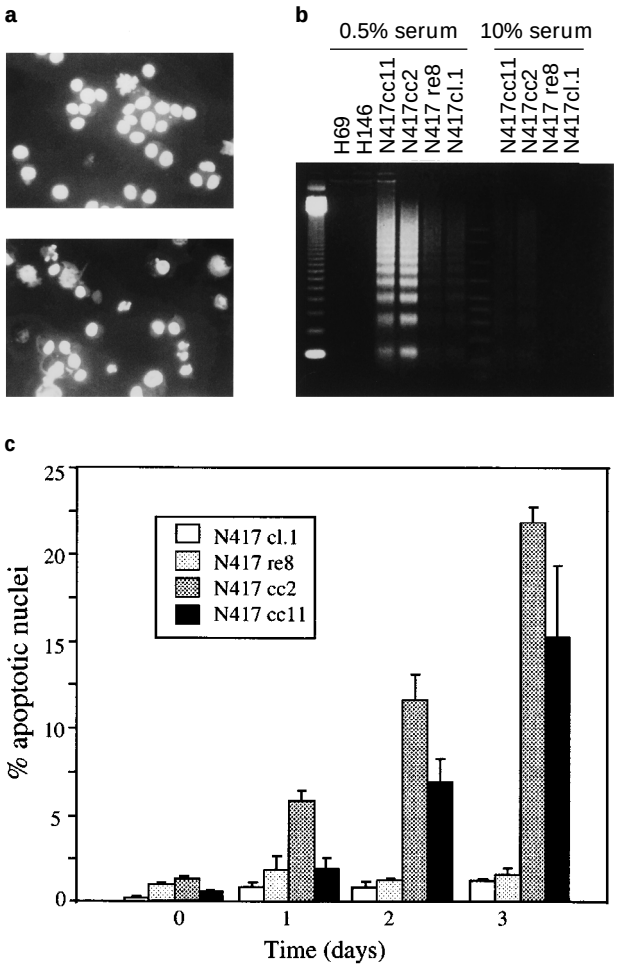

Figure 5 Apoptosis of N417 clones expressing CC3 in medium with low serum content. (**a**) Hoechst 33342 staining of the nuclei of N417ccre8 cells (top) and N417cc2 cells (bottom) incubated in medium with 0.5% serum for 72 h. (**b**) DNA fragmentation analysis of cells incubated either in complete or low serum medium for 72 h. Unmarked lanes contain DNA ladders of 100 and 123 base pairs. (**c**) Percent of apoptotic cells in serum-starved cultures was determined by ultraviolet microscopy of cell nuclei stained with Hoechst 33342. All determinations were performed independently three or four times with at least 500 nuclei counted each time. Bars, standard deviations

clones decreased significantly faster and to a greater degree than that of control clones. The vast majority of N417cc2 and N417cc11 cells died within 8 to 10 days after shift to the medium with low serum content (Figure 4). Cultures of control clones maintained a relatively high percentage of live cells (Figure 4); moreover, the viable cells in serum-starved control cultures continued to proliferate, so that the absolute numbers of cells increased requiring regular passage of cultures. The viability of about 50% and continuous proliferation were maintained in control clones even after prolonged incubation of several weeks (data not shown). Thus, the loss of viability in N417 cultures deprived of serum is counterbalanced by continuous proliferation of surviving cells. However, the massive death of cells in serum-starved cultures of N417 clones expressing CC3 results in the collapse of these cultures. Comparison of the agarose colony formation by control and experimental clone under low serum conditions showed a 10-fold reduction in number of colonies (not shown). These results demonstrate that introduction of CC3 into N417 cells greatly reduces their ability to survive growth factor withdrawal *in vitro*.

Examination of the nuclear morphology of N417cc2, N417cc11 and N417cc12 cells from serum starved cultures revealed presence of picnotic and fragmented nuclei typical for apoptotic cells (Figure 5a), suggesting that apoptotic mechanisms are involved in death of these cells. Percent of apoptosis was calculated by counting the relative numbers of nuclei displaying apoptotic morphology. This quantitative analysis indicated that proportion of apoptotic cells progressively increases in serum starved CC3 expressing cells (Figure 5c). Though a low percentage of apoptotic cells (1–3% of total) could be detected in control clones, it did not increase significantly as a function of time (Figure 5c). The extent of apoptosis was independently quantitated through detection of apoptotic cells by specific 3′ end labeling of DNA ends *in situ* (Gavrieli *et al*., 1992). Percentage of apoptotic cells identified by this method was generally somewhat higher than that measured by nuclear morphology (data not shown). Additionally, apoptosis of CC3 expressing clones was confirmed by demonstration of abundant extrachromosomal fragmented DNA in serum starved N417cc2 and N417cc11 cells (Figure 5b).

Very similar results were obtained when CC3 was introduced into an additional highly metastatic v-SCLC cell line H82. H82cc clones expressing exogenous CC3 were subject to rapid loss of viability upon serum withdrawal; many cells in the serum-starved H82cc populations had picnotic and fragmented nuclei. Quantitation of apoptotic cells in the experimental and control clones of H82 by staining for 3′ DNA ends revealed that in clones expressing CC3 serum starvation induces apoptosis of 8–12% cells within 3 days, while only 2–4% of cells were apoptotic in control clones.

Response to serum starvation was examined in SCLC cell lines known to express CC3 RNA: c-SCLC

cell lines H146 and H69 and an atypical v-SCLC line H446 that has a relatively low metastatic ability in SCID-hu mice (Shtivelman and Namikawa, 1995). Incubation of these cell lines in medium with 0.5% serum did not induce apoptosis by any of the following criteria: nuclear morphology (less than 2% of nuclei were picnotic or fragmented); DNA fragmentation (Figure 5b) or staining for the 3′ DNA ends (not shown). However, cell lines H146 and H446 lost more than 90% of their viability within one week of incubation in low serum. Loss of viability in H69 cultures was more gradual, resulting in collapse of cultures within 2 to 3 weeks. Apparently expression of CC3 in c-SCLC lines does not predispose them to apoptotic death possibly due to presence of anti-apoptotic activities within these cells.

The ability of CC3 to induce apoptosis *in vivo* was examined by identification and counting apoptotic cells in tumors induced by N417 cells expressing CC3 and by control N417 clones in the HFL grafts of SCID-hu-L mice. Tumor sections were processed for the *in situ* 3′ end labeling of DNA. Quantitation of apoptotic cells showed that the incidence of apoptotic cells in CC3 expressing tumors is three- to eightfold higher than in tumors induced by control N417ccre clones (Figure 6). Thus, expression of CC3 in v-SCLC cells predisposes them to apopotosis both *in vitro* and *in vivo*.

V-SCLC are generally more resistant than c-SCLC to killing by cytotoxic agents (Carney *et al*., 1983; Aisner *et al*., 1990). Control and CC3 expressing clones of N417 cells were exposed to VP16 and cisplatin at doses that do not cause significant killing of N417 cells. Both drugs induced significant loss of viability in treated N417cc clones (data not shown) and an increase in proportion of apoptotic cells (Figure 7). H82 cells modified to express CC3 showed a similar increase in chemosensitivity (data not shown).

A potential mechanism for CC3 induced apoptosis could be down regulation of bcl-2, a protein preventing apoptosis in a wide variety of normal and malignant cells (Reed, 1995). bcl-2 was shown previously to be expressed in several SCLC cell lines (Ikegaki *et al*., 1994). Examination of bcl-2 expression in control and CC3 expressing N417 clones revealed absence of bcl-2 mRNA and protein (data not shown). bcl-2 RNA has been examined in additional SCLC lines with known metastatic potential in the SCID-hu-L mice. High levels of bcl-2 RNA were found in five out of five c-SCLC lines examined (three of them shown in Figure 8) and a lower level in v-SCLC H446; practically no bcl-2 transcript was detectable in two highly metastatic v-SCLC lines H82 and N417 (Figure 8). bcl-2 protein was highly expressed in c-SCLC cell lines and at appreciable levels in H446 (data not shown) and its presence might contribute to the relative resistance to apoptosis of c-SCLC lines and H446. Lack of CC3 in metastatic v-SCLC cells might protect them from apoptosis even in the absence of bcl-2 protein and at the same time play a role in their high metastatic ability.

Discussion

Several metastasis-suppressor genes have been identified, among them NM23 (Leone *et al*., 1991) for breast and colorectal cancer, E-cadherin for epithelial cancers

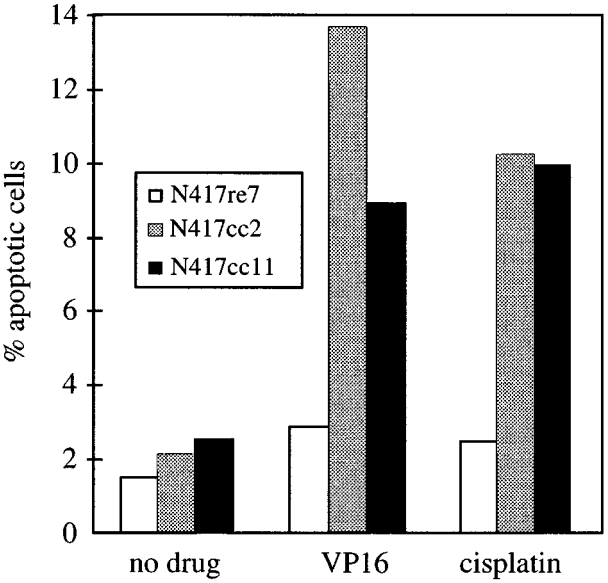

Figure 7 Apoptosis of N417cc clones treated with cytotoxic drugs. Cells were treated for 24 h with 50 μg/ml of VP16 or 5 μg/ml of cisplatin. Number of apoptotic cells was assessed using the Apoptag Plus kit (Oncor). The results are average of at least two independent experiments

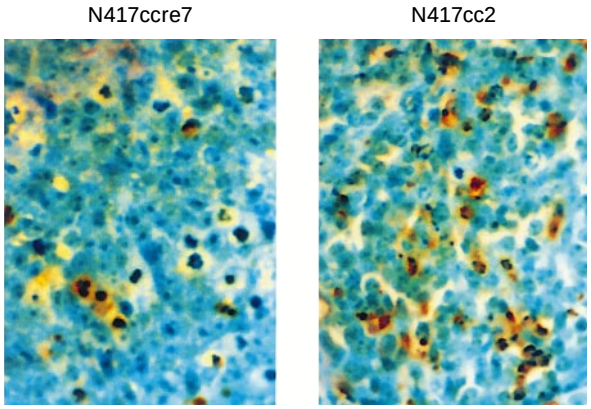

Figure 6 Apoptosis of N417cc cells *in vivo*. Sections of tumors induced by direct injection of indicated cell lines into HFL grafts were stained for apoptotic cells using the Apoptag Plus kit (Oncor). Sections were counterstained with Methyl green

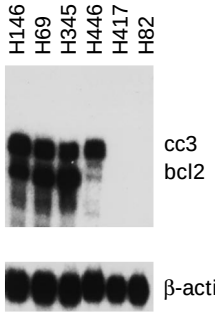

Figure 8 Analysis of bcl-2 RNA in SCLC cell lines. The RNase protection analysis was performed as described in legend to Figure 3. Three probes were used: bcl-2 (BamHI–PstI fragment of 253 nucleotides from a cDNA clone of bcl-2), CC3 and β-actin (as in Figure 3)

(Birchmeier and Behrens, 1994), and KAI1 for prostate carcinoma (Dong *et al*., 1995). While the mechanism of metastasis suppression by NM23 is unknown, the latter two genes encode cell membrane proteins, whose expression presumably modifies the adhesive and invasive characteristics of tumor cells. The candidate metastasis-suppressor gene CC3 described here affects a more basis characteristic of tumor cells, ie their ability to die. Introduction of CC3 into highly metastatic v-SCLC cells results in suppression of metastases *in vivo*, massive cell death under conditions of serum deprivation *in vitro*, and increased sensitivity to cytotoxic drugs.

V-SCLC cells might be actually predisposed to apoptosis due to the following: they express high levels of c-*myc* (Little *et al*., 1983), which was shown to promote apoptosis (Evan *et al*., 1992; Shi *et al*., 1992) and lack *bcl-2* protein (Ikegaki *et al*., 1994) that is capable of suppressing the death-promoting activity of c-*myc* (Bissonnette *et al*., 1992; Fanidi *et al*., 1992). In addition, most SCLC cell lines contain high levels of bax protein (Reeve *et al*., 1996) that is a positive regulator of apoptosis. Apparently a factor other than *bcl-2* confers a survival advantage on v-SCLC cells. Loss of CC3 expression might be such a factor. It is of interest that the c-SCLC cell lines that are also derived from metastatic tumors express both *bcl-2* and CC3 (Ikegaki *et al*., 1994, and my data). Metastasis of v-SCLC might be enhanced by their ability to resist apoptosis due to the lack of CC3 expression; metastatic c-SCLC cells might protect themselves from CC3-induced apoptosis by expressing high levels of bcl-2.

The results presented here establish a link between the high metastatic potential of v-SCLC cells and their ability to resist apoptotic signals. Conceptually this link is almost obvious: metastatic cells have to be able to withstand the severe pressures of metastatic cascade and preserve viability under conditions that would cause its loss in non-metastatic cells. The ability of a tumor cell to resist death signals might be more important for the successful metastatic event than it is in the context of a large primary tumor. Metastatic cells should be able to survive and proliferate in order to form a micrometastatic lesion that can persist in spite of initial lack of vascularization and growth factor deprivation (Folkman, 1990). Recent findings on the dormancy of micrometastasis show that in the absence of angiogenesis apoptosis suppresses overt growth of micrometastases (Holmgren *et al*., 1995). The state of dormancy is a result of balance between cell proliferation and apoptosis occurring in micrometastases. Lack of CC3 in v-SCLC and potentially its inactivation in other metastatic tumors might change the balance in favor of proliferation by suppressing apoptosis induced by lack of growth factors.

The demonstration of the role that resistance to apoptosis plays in metastasis implies that successful metastatic cells might be more resistant to cytotoxic treatments such as radiation and chemotherapy. Indeed, introduction of CC3 into v-SCLC cells is capable not only to suppress their metastasis but also induces relative chemosensitivity thus linking two important clinical features of v-SCLC: aggressive metastatic spread and drug resistance.

Levels of CC3 RNA greatly vary from one tumor cell line to another even within same histologic type (not shown). It is possible that these quantitative differences play a role in the susceptibility of tumor cells to the apoptotic stimuli and in their metastatic potential. Analysis of the levels of CC3 and known death-regulatory proteins might be predictive of the degree of aggressiveness of a given tumor. Other than v-SCLC subset of tumor cell lines that completely lack CC3 expression are neuroblastoma lines derived from the late stage highly disseminated tumors. It is possible that lack of CC3 in tumors of neuroendocrine origin or tumors with a neuroendocrine component plays a role in their metastatic phenotype.

Materials and methods

Cell culture and transfection

Cell lines were obtained from American Type Cell Collection and cultured in RPMI 1640 medium supplemented with 10% of fetal calf serum. Cell lines N417, H345, H146 and H69 grow as suspension cultures, H82 cells are semi-adherent and H446 cells are adherent. CC3 expression constructs were introduced into N417 and H82 cells by electroporation.

Animal studies

C.B-17 *scid/scid* mice were used at the age of 6–8 weeks. The human fetal tissues were obtained with informed consent according to regulations issued by each state and by the federal government. Construction of SCID-hu-L mice was described previously (Shtivelman and Namikawa, 1995). Briefly, $2 \times 2 \times 2$ mm fragments of human fetal lung of 18–22 gestational week were implanted into fourth mammary pads of SCID mice; resulting SCID-hu-L mice were used for experiments between 4 to 8 weeks after engraftment. For experimental metastasis assay collected cells were counted and suspended in Hanks balanced salt solution (HBSS) supplemented with 2% fetal calf serum. Only cell preparations with viability of more than 85% were used for the *in vivo* experiments. Cells were injected intravenously through the tail vein into SCID-hu-L mice in a volume of 0.2 ml per animal. Mice were euthanized at the termination of experiments by CO_2 inhalation. Mouse lungs and human tissue grafts of all experimental animals were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin according to standard histologic procedures. Sections were subjected to microscopic examination to confirm or rule out the presence of tumors.

RNA analyses

Total RNA was extracted from actively growing cells. Differential display of RNA was performed as described (Liang and Pardee, 1992) using the kit from GeneHunter Corp. The 5′ end of CC3 cDNA was obtained with the 5′-RACE kit from Clontech Inc. and CC3-specific nested primers according to manufacturer protocols. Primer sequences were derived from the sequence of the original cDNA fragment obtained through differential display and were as follows: GCCATGCGCTTTCCCCAGGTCATG-GATGG (nucleotides 784–812 in the sequence of CC3 deposited in the GenBank) and CTCGCTCGCCCATTG-TCTCTTGGTCTCACCACATTGTTCAGC (nucleotides 728–757 and additional sequences for cloning into the plasmid vector pCRDirect (Clontech, Inc.). RNase protection analysis was performed using reagents from Ambion Inc.

Growth curves and soft-agar clonogenic assay

Experimental and control clones of N417 were plated at $10^5 - 2 \times 10^5$ cells per ml of medium in duplicate. Live cell concentration was determined by counting in hemacytometer with Trypan blue at 24 h intervals. All experiments were performed at least three times. Doubling times were calculated from the linear portions of the semi-logarithmic growth curves. For the soft agarose assays cells were plated at 5×10^3 per well in six well plates in 0.3% agarose over a 0.5% agarose layer. Colonies of more than about 50 cells were counted 13 days after seeding. All experiments were in triplicates and repeated at least twice.

DNA fragmentation assay

Cells were lysed in hypotonic buffer (10 mM Tris, 10 mM EDTA, pH 8.0) containing 0.2% Triton X-100 (Sellins, 1987). The nucleic acids in the lysate were extracted with phenol-chloroform:isoamyl alcohol, concentrated by ethanol precipitation, treated with RNase A and electrophoresed in 2% agarose gel.

3′ end labeling of DNA in apoptotic cells

The 3′ end labeling of DNA *in situ* was performed using the ApopTag Plus kit (Oncor). Cells grown *in vitro* were spun onto glass slides and fixed with buffered 4% formaldehyde. The manufacturer suggested protocol for detection of DNA breaks was followed both for the paraffin tissue sections and for the cytospin preparations.

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References

Aisner SC, Finkelstein DM, Ettinger SD, Abeloff MD, Ruckdeschel JC and Eggleston JC. (1990). *J. Clin. Oncol.*, **8,** 402 – 408.
Birchmeier W and Behrens J. (1994). *Biochimica et Biophysica Acta*, **1198,** 11 – 26.
Bisonnette R, Echeverri F, Mahboubi A and Green DR. (1992). *Nature*, **359,** 552 – 554.
Bunn PA, Linnoila I, Minna JD, Carney D and Gazdar AF. (1985). *Blood*, **65,** 764 – 768.
Carney DN, Mitchell JB and Kinsella TJ. (1983). *Cancer Res.*, **43,** 2806 – 2811.
Carney DN, Gazdar AF, Bepler G, Guccion JG, Marangos PJ, Moody TW, Zweig MH and Minna JD. (1985). *Cancer Res.*, **45,** 2913 – 2923.
Carney DN and De Leij L. (1988). *Sem. in Oncology*, **15,** 199 – 214.
Dearing MP, Steinberg SM, Phelps R, Anderson MJ, Mulshine JL, Ihde DC and Johnson BE. (1990). *J. Clin. Oncology*, **8,** 1042 – 1049.
Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Issacs JT and Barret JC. (1995). *Science*, **268,** 884 – 886.
Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell*, **69,** 119 – 128.
Fanidi A, Harrington EA and Evan GI. (1992). *Nature*, **359,** 554 – 556.
Folkman J. (1990). *J. Natl. Cancer Inst.*, **82,** 4 – 6.
Frohman MA, Dush MK and Martin GR. (1988). *Proc. Natl. Acad. Sci. USA*, **85,** 8998 – 9002.
Gavrieli Y, Sherman Y and Ben-Sasson SA. (1992). *J. Cell Biology*, **119,** 493 – 501.
Gazdar AF, Carney DN, Nau MN and Minna JD. (1985). *Cancer Res.*, **45,** 2924 – 2930.
Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, Schuller HM and Park JG. (1988). *Cancer Res.*, **48,** 4078 – 4082.
Holmgren L, O'Reilly MS and Folkman J. (1995). *Nature Med.*, **1,** 149 – 153.
Ikegaki N, Katsumata M, Minna J and Tsuimoto Y. (1994). *Cancer Res.*, **54,** 6 – 8.
Jensen SM, Gazdar AF, Cuttitta F, Russell EK and Linnoila RI. (1990). *Cancer Res.*, **50,** 6068 – 6074.
Leone A, Flatow U, King CR, Sandeen MA, Margulies IMK, Liotta LA and Steeg P. (1991). *Cell*, **65,** 25 – 35.
Liang P and Pardee AB. (1992). *Science*, **257,** 967 – 970.
Little CD, Nau MM, Carney DN and Minna JD. (1983). *Nature*, **306,** 194 – 196.
Morstyn G, Russo A, Carney DN, Karawya E, Wilson SH and Mitchell JB. (1984). *J. Natl. Cancer Inst.*, **73,** 801 – 807.
Radice PA, Matthews MJ, Ihde DC, Gazdar AF, Carney DN, Bunn PA, Cohen MH, Fossieck BE, Makuch RW and Minna JD. (1982). *Cancer*, **50,** 2894 – 2902.
Reed JC. (1995). *Curr. Opinion in Oncology*, **7,** 541 – 546.
Reeve JG, Xiong J, Morgan J and Bleehen NM. (1996). *Br. J. Cancer*, **73,** 1193 – 1200.
Sellins KS and Cohen JJ. (1987). *J. Immunology*, **139,** 3199 – 3206.
Shi Y, Glynn JM, Guilbert LJ, Cotter TG, Bissonnette RP and Green DR. (1992). *Science*, **257,** 212 – 214.
Shtivelman E and Namikawa R. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 4661 – 4665.
Sieffer EJ and Ihde DC. (1988). *Semin. Oncol.*, **15,** 278 – 299.
Weidenmann B, Franke WW, Kuhn C, Moll R and Gould VE. (1986). *Proc. Natl. Acad. Sci. USA*, **83,** 3500 – 3540.