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ARTICLES

Cutaneous Rat Wounds Express *C49a,* a Novel Gene With Homology to the Human Melanoma Differentiation Associated Gene, *Mda-7*

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Abstract We have used DD-PCR (differential display-polymerase chain reaction) to identify new genes that are over- or underexpressed during wound repair. DD-PCR performed on excisional wounds identified the expression of rat *c49a.* Cloning and sequence analysis of the rat *c49a* gene revealed high homology to a novel human melanoma differentiation associated gene, *mda-7.* The human *mda-7* gene isolated from melanoma cell lines, has been linked with human melanoma differentiation, and growth suppression. Moreover, transfection of human *mda-7* constructs into human tumor cells suppresses the growth and colony formation of tumor cells from diverse origins. To confirm and relatively quantitate expression of rat *c49a* gene during repair, specific primer, reduced cycle RT-PCR (reverse transcription-PCR) was performed. RT-PCR showed an ~9 to 12-fold elevation of rat *c49a* mRNA at 12 h to 5 days above nonwounded controls that gradually decreased to ~1.5 to 3-fold by day 14. Cloning and sequence analysis of the entire 1200 base pair *c49a* gene product showed 78% nucleotide homology to human *mda-7.* Immunohistochemistry studies localized rat *C49A* expression primarily to fibroblast-like cells at the wound edge and base. The marked up-regulation of rat *c49a* transcripts during the inflammatory and early granulation tissue phases of wound repair where cellular processes such as re-epithelialization, angiogenesis, and fibroplasia predominate—suggest that *c49a* is associated with proliferation of fibroblasts in wound healing. J. Cell. Biochem. 74:1–10, 1999.

Key words: cell proliferation; differential display; fibroblasts; gene expression; growth suppression; wound edge; wound healing

Wound healing, embryogenesis, and tissue regeneration are all processes that require regulated cellular proliferation and differentiation [Stocum, 1995]. Tumorigenesis, on the other hand, represents a loss of control over cell growth and differentiation. At the most simplistic level, regulation of proliferation and differentiation involves finely orchestrated interactions among different cell types (and cell differentiation states), extracellular matrix (ECM) molecules, and growth factors or cytokines. At a more complex level, this entails multilevel cellular transcriptional and translational control of the downstream effector molecules that respond to the changes in growth factor secretion and/or ECM composition. Many protooncogenes, tumor suppressor genes, cell cycle regulator genes [reviewed in Henriksson and Luscher, 1996], as well as homeobox genes [Duboule, 1994] have been implicated in the downstream molecular events that fundamen-

Abbreviations used: BLAST, Basic Alignment Search Tool; *c49a*, *c49a* transcript; *C49A*, *C49A* protein; d, day; DD-PCR, differential display-polymerase chain reaction; ECM, extracellular matrix; FAS, Freund's Adjuvant Serum; HPV, human papillomavirus; mda-7, melanoma differentiation associated gene transcript; MDA-7, melanoma differentiation associated gene protein; NCBI, National Center for Biotechnology Information; nt, nucleotide; PBS, phosphate buffered saline; RACE, Rapid Amplification of cDNA Ends; RT-PCR, reverse transcription-polymerase chain reaction.

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tally govern cellular fate; however, much remains unknown.

To better understand the cellular processes that control wound repair, we analyzed gene expression by differential display in a rat cutaneous wound model. From this, we have isolated a rat sequence (*c49a*) with homology to human *mda-7,* a novel melanoma differentiation associated gene [Jiang et al., 1995]. Prior studies have demonstrated that human *mda-7* is induced in human melanoma cells pharmacologically manipulated to undergo growth arrest and terminal differentiation [Jiang et al., 1995]. Moreover, transfection of human *mda-7* constructs into human melanoma and diverse human cancers of breast, central nervous system, cervix, colon, prostate, and connective tissue origin, suppresses the growth and colony formation of these cancer cells [Jiang et al., 1996]. These data, taken together with the fact that the human *mda-7* sequence is present among such divergent species as humans, primates, and yeast, suggest that *mda-7* is an evolutionary conserved sequence with growth inhibiting properties in human cancers with multifactorial genetic defects [Jiang et al., 1996].

In the present study, we demonstrate the identification, full-length isolation, and immunohistochemistry localization of rat *c49a,* a gene with homology to human *mda-7.* We also show the marked up regulation of rat *C49A* protein expression by spindle-shaped cells at the base and edge of the wound during cutaneous repair. The temporal expression of rat *c49a* RNA and protein corresponds to the inflammatory and early granulation tissue phases of wound healing where cellular processes such as re-epithelialization, angiogenesis, and fibroplasia predominate [Clark, 1993; Kirsner and Eaglstein, 1993]. Our results suggest that rat *c49a* may be a member of the *mda-7* gene family rather than the rat homologue of human *mda-7,* and that rat *c49a* may be associated with proliferation rather than growth suppression.

MATERIALS AND METHODS

Animal Wounding, Tissue Harvest I, and Preparation of Wound Tissues

To assess wound edge gene expression during cutaneous repair, 40 male Sprague-Dawley rats (300–350 g; Group I) were anesthetized with a single intraperitoneal injection of 45 mg/kg pentobarbital and shaved. To minimize regional cephalocaudal and dorsoventral differences in wound healing, spatial landmarks such as the forelimb and hindlimb articulation sites as well as the spine were used to consistently mark excisional wound locations [Auerbach and Auerbach, 1982]. Eight full-thickness, excisional skin wounds including the panniculus carnosus were made (7 mm^2; n = 280 wounds) on the backs of 35 animals. All wounds were separated by at least 2 cm to eliminate effects from neighboring wounds. Five animals at a time were re-anesthetized and the isolated wound edge excised in a picture-frame pattern with a 3 mm margin at 12 h, 24 h, 3 d, 5 d, 7 d, 10 d, and 14 d after injury. Controls consisted of nonwounded skin (0 h) from identical locations in five animals. To minimize possible wound healing responses to shaving with an electric clipper, nonwounded control animals underwent skin harvest within 1 min of hair removal. Wound tissue from each time point was immediately frozen in liquid nitrogen, combined, and stored at −70°C until RNA isolation.

Tissue Harvest II

To determine if there are differences in gene expression between wound edge skin and the entire wound, a second group of 20 rats was anesthetized. Sixteen animals were then wounded in an identical fashion as Group I animals (7 mm^2 wounds; eight wounds/animal; n = 128 wounds). Two animals at a time were re-anesthetized and all eight wounds on each animal were excised in their entirety (including the base) at 12 h, 24 h, 2 d, 3 d, 5 d, 7 d, 10 d, and 14 d after injury. Six wounds from each animal were excised with a 3 mm margin for RNA isolation, while the two most cephalad wounds were excised with a 5 mm margin for histology. Controls consisted of nonwounded skin (0 h) from identical locations in four animals.

RNA Isolation

Total RNA was extracted in a mono-phasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent; Gibco BRL, Gaithersburg, MD). Relative RNA integrity was then assessed by fractionating 2 μg of total RNA in a glyoxal/dimethyl sulfoxide gel and visualizing the 28S and 18S ribosomal RNA bands [Sambrook et al., 1989].

Differential Display Polymerase Chain Reaction

Differential Display Polymerase Chain Reaction (DD-PCR) was performed using arbitrary

primer sequence sets from the RNAimage™ Kit (GenHunter Corp., Nashville, TN). The DD-PCR products were separated on 6% acrylamide, 8 M urea gels, and analyzed by autoradiography. Differentially up- or downregulated gene fragments were extracted from the gel and reamplified using the same set of DD-PCR primers and cloned into the pCR™ 2.1 vector using the TA Cloning® Kit (Invitrogen, San Diego, CA). Plasmids from positive colonies were purified and sent to the DNA Sequencing Facility at UCLA. The cDNA sequences were analyzed using BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information).

Specific Primer RT-PCR and Southern Blotting of PCR Products

To verify the differential display expression pattern for rat *c49a*, specific primer sequence sets were designed for the rat *c49a* cDNA fragment isolated from DD-PCR (Forward primer: 5′ ACCTTTGGATGCTCCGACTGAC 3′; Reverse primer: 5′GGAAGTAAG-GTGCAAACAC-GGG 3′). Specific primer reverse transcription (RT)-PCR was then performed. PCR was carried out at 30, 25, 20, and 18 cycles. Primers specific for GAPDH were used as an internal control. The PCR products were electrophoresed and transferred to nylon membranes. Correct PCR product amplification was confirmed by probing the nylon membrane with a ^{32}P-labeled oligonucleotide specific for a region of the PCR amplified rat *c49a* cDNA fragment (5′ CGGTTTCTACCGAA-ACAGTGAC 3′). Hybridized membranes were exposed to Biomax films with an intensifying screen (Kodak, Rochester, NY) at −70°C for 24 h. Autoradiograms were scanned and analyzed on a Macintosh computer using the public domain NIH Image program.

Rapid Amplification of cDNA Ends

Isolation of the full-length rat *c49a* cDNA clone was accomplished by 5′ Rapid Amplification of cDNA Ends (RACE; Marathon™ cDNA Amplification Kit, Clontech, Palo Alto, CA). First strand cDNA synthesis was performed with 5 µg of total rat RNA from 12 h wounds using an oligo dT(30-mer) primer. Second strand synthesis was performed with RNAse H, *E. coli* DNA ligase, and *E. coli* DNA polymerase. T4 DNA polymerase was added to create blunt ends on the double stranded cDNAs prior to adapter ligation. Double stranded cDNAs were then ligated to the 5′-cDNA adapter: 5′ CTAATAC-GACTCACTATAGGGCTCGAGCGGCCGCC-CGGGCAGGT 3′ with T4 ligase. This product was then amplified by PCR using the 5′ AP1 primer (a 27-mer complementary to the 5′-cDNA adapter) and a 3′ sequence specific reverse primer (a 26-mer designed from the rat *c49a* clone): 5′ CAGGAAGTAAGGTGCAAACA-CGGGGC 3′. Nested PCR was then performed with 0.1 µL of the previous PCR product using the 5′ AP2 primer (a 22-mer from the 3′ end of the cDNA adapter) and a second 3′ rat *c49a* specific reverse primer (a 23-mer 95 bases upstream of the first sequence specific primer): 5′ CCACGTTGTGGGTCAGTCGGAGC 3′. The size of the PCR products were analyzed on an agarose gel and correct product amplification confirmed by Southern blotting and probing with the P^{32}-labeled rat *c49a* cDNA fragment (isolated from DD). The PCR products were then run on a second gel and the appropriately sized fragment excised, purified, and cloned using the TA Cloning® Kit (Invitrogen). Plasmids from positive colonies were purified and sent to the DNA Sequencing Facility at UCLA. The cDNA sequences were analyzed using BLAST at NCBI and ALIGN at Human Genome Center, Baylor College of Medicine.

C49a Peptide Polyclonal Antibodies

The polypeptide MLPISDSARRRFLLF, corresponding to amino acids 136 to 150 of *C49A* was generated using the PCGENE program. The polypeptide was then conjugated to keyhole limpet hemacyanin (Calbiochem, San Diego, CA). Two hundred µg of the peptide conjugate in complete Freund's Adjuvant Serum (FAS) was injected into each of two rabbits (New Zealand white, female, Hazelton Labs, Denver, PA). Subsequent 100 µg inoculations of the peptide conjugate in incomplete FAS were given at 2 week intervals for three times. All inoculations were given subcutaneously at two sites. Sera were collected at 7, 9, and 11 weeks with the final bleed at 11.5 weeks. An 100 µg booster of peptide conjugate in incomplete FAS was given 1 week before the second and third sera collection. ELISA analyses were performed on the final bleed sera for the presence of an anti-peptide response. The anti-peptide titer was defined as the dilution with an OD ($>$0.1) above background, and was determined to be 1:15,000.

Immunohistochemistry

Eight μm paraffin sections of 2-day-old wounds were made and mounted on Vectabond prep slides (Vector Lab, Burligame, CA). Deparaffinized sections were treated with xylene and rehydrated with phosphate buffered saline (PBS). This was followed by the addition 0.1% hydrogen peroxide in methanol at room temperature for 10 min to block endogenous peroxide. After treatment with PBS buffer, sections were blocked with 0.5% BSA in PBS at room temperature for 30 min in a humidity chamber. After draining the slides, 1:500 dilution of anti-*C49A* antibody with PBS and 0.1% BSA were applied and incubated at 4°C for overnight in a humidity chamber. After rinsing with PBS, a goat anti-rabbit biotinylated secondary antibody of 1:1,000 dilution was used with a standard avidin-biotin complex/immunoperoxidase protocol (Vector Elite Kit, Vector Lab). Diaminobenzidine peroxidase substrate was used and counterstained with hematoxylin. Controls consisted of pre-immune rabbit sera at 1:500 dilution on 2 day wounds, and *C49A* sera at the same dilution on nonwounded skin.

RT-PCR Cloning

Human *mda-7* was originally isolated by Jiang et al. [1995] from a melanotic melanoma cell line, HO-1. To determine if there are nucleotide sequence differences between human melanoma *mda-7* and *mda-7* from nonmalignant, nonmelanocyte cell types, we isolated human *mda-7* from a human papillomavirus (HPV) transformed human keratinocyte cell line, HOK [Park et al., 1991; Shin et al., 1994]. Primers for RT-PCR were designed to specifically amplify a 1134 nucleotide long sequence of human *mda-7* from position 75 to 1208 that encompasses the human *mda-7* coding region (Forward primer: 5′ TGATTGGTGAATGGTGAAGG 3′; Reverse primer: 5′ TTCTGCTGCCTTCATGTAAC 3′). The PCR products were then cloned using the TA Cloning® Kit (Invitrogen). Plasmids from positive colonies were purified and sent to the DNA Sequencing Facility at UCLA. The cDNA sequences were analyzed using BLAST at NCBI and ALIGN at Human Genome Center, Baylor College of Medicine.

RESULTS

Expression of Rat *C49a* During Wound Repair

To better understand the molecular control of wound healing, DD-PCR was used to identify potentially new genes that may be up or down regulated by repair. DD-PCR was performed on wound edge (Tissue harvest I) RNA isolated from rat excisional skin wounds at 12 h, 24 h, 3 d, 5 d, 7 d, 10 d, and 14 d after acute injury and from 0 h (nonwounded controls). One 260 nucleotide (nt) fragment, *c49a*, on DD-PCR showed significant upregulation beginning 12 h postwounding (Fig. 1). This gene upregulation was maintained for at least 7–10 days and was followed by a gradual decline towards baseline by 14 days.

Cloning and sequence analysis of the rat 260 nt cDNA fragment revealed high homology [$1.3e^{-19}$ P(N)-probability by chance on BLAST] to a novel human melanoma differentiation associated gene, *mda-7*. The rat *c49a* 3′ differential display fragment aligned to the complete human *mda-7* cDNA of 1700 nt at 1063-1205 nt. Since the coding sequencing for human *mda-7* is only from 275-895 nt, we concluded that the human *mda-7* sequence may have a longer 3′ untranslated region than rat *c49a*. Thus, the

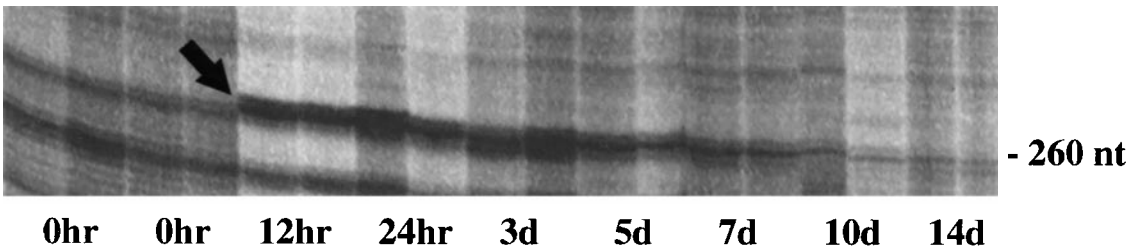

Fig. 1. Differential display results from full-thickness excisional rat skin wounds at time 0 to 12 h, 24 h, 3 d, 5 d, 7 d, 10 d, and 14 d after wounding. DD-PCR reactions from each time point were performed in duplicate as described in the Methods section. The arrow indicates the elevated expression of an ~260 nucleotide differential display product starting at 12 h, with maintained overexpression until day 7, followed by a gradual return to baseline by day 14. Time 0 represents normal, nonwounded skin.

predicted size for the full-length rat *c49a* cDNA should be ~1200 nt.

To confirm and relatively quantitate expression of the rat *c49a* gene during repair, we used the sequence obtained from the 260 nt rat *c49a* differential display fragment to design two rat *c49a* specific primers for reduced cycle RT-PCR. The PCR product after gel electrophoresis and Southern blotting was then probed with a third rat *c49a* sequence specific oligonucleotide (Fig. 2). Eighteen cycles was the minimum number of PCR cycles detectable at 24 h exposure. A similar pattern of *c49a* induction was observed from 18 to 25 cycles. A lesser degree of induction was observed at 30 cycles, suggesting nonlinear amplification in the PCR curve. Specific primer RT-PCR showed an ~9 to 12-fold elevation of rat *c49a* mRNA at 12 h to 5 days above nonwounded controls that gradually decreased to ~1.5 to 3-fold by day 14. RNA extracted from wound edge (Tissue Harvest I) and entire wound (Tissue Harvest II) displayed slightly different kinetics of *c49a* expression by RT-PCR (Fig. 2). No significant change in expression was observed for the GAPDH controls (Fig. 2).

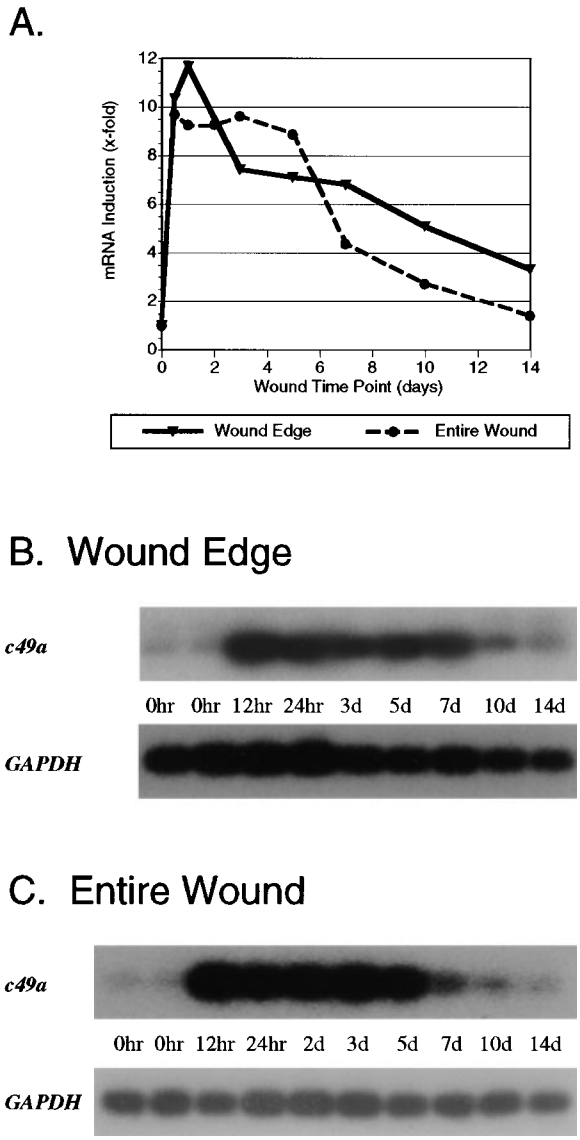

Fig. 2. Reduced cycle RT-PCR determination of rat *c49a* expression in excisional wounds. Specific 5′ and 3′ primer sequences were designed from the original 260 nucleotide rat *c49a* fragment isolated from differential display. Reduced cycle RT-PCR was then performed on 1µg of RNA isolated from either wound edge (Tissue harvest I) or entire wound (Tissue harvest II). The expected 152 base pair PCR product was electrophoresed, Southern blotted, and then probed with an internal oligonucleotide sequence. The autoradiograms were scanned and the induction of *c49a* mRNA, relative to non-wounded controls (Time 0), calculated. Each experiment was performed in duplicate and the x-fold mRNA induction values averaged and depicted graphically. A representative autoradiogram is shown for each wound edge or entire wound RT-PCR for rat *c49a* and GAPDH controls.

Full Cloning and Initial Characterization of Rat *C49a*

In order to confirm the homology between rat *c49a* and human *mda-7*, the full-length rat *c49a* cDNA was cloned by 5′RACE performed on 12 h rat wound RNA. This produced a distinct band size of approximately 1200 base pairs that hybridized with the initial rat *c49a* cDNA fragment isolated from DD-PCR. The ~1200 base pair PCR product was then gel purified and cloned into the pCR™ 2.1 vector using the TA Cloning® Kit (Invitrogen). Plasmids from positive colonies were purified, sequenced, and analyzed using BLAST. Two clones with identical 5′ ends showed significant homology to human *mda-7* [$2.7e^{-159}$ P(N)]. The rat *c49a* cDNA was 1107 nt in length with 71.7% nucleotide homology to human *mda-7* in the coding region. This homology between rat *c49a* and human *mda-7* increased to 82.0% homology in a conserved 492 nt coding segment (Fig. 3). Like human *mda-7*, the 3′ untranslated region of rat *c49a* also contained copies of the AUUUA sequence motif involved in mRNA destabilization (Fig. 3) [reviewed in Schiavi et al., 1992].

Analysis of the rat *c49a* nucleotide sequence revealed several possible open reading frames, but only one with a Met start codon (Codon Usage, University of Minnesota). If Met was designated the initiator codon, then rat *c49a* had a predicted open reading frame that started at nucleotide position 181 and terminated at position 732, encoding a 183 amino acid, 21.1

Fig. 3. Global alignment of rat *c49a* and human *mda-7* coding sequences by ALIGN at EERIE, Nimes, France. The entire rat *c49a* sequence and the human *mda-7* coding region is shown. Rat *c49a* coding region is from 181-732 nt, while human *mda-7* is from 275-895 nt. There is 71.7% global homology between coding sequences for rat *c49a* and human *mda-7* with 82% homology is a highly conserved 492 nt segment (single underline). The four rat *c49a* 3′ untranslated consensus sequences (AUUUA) for mRNA destabilization are shown (double underline). These sequence data are available from GenBank/EMBL/DDBJ under accession number AF004774.

kDa polypeptide. In contrast, human *mda-7* (also with a Met codon initiator) encoded a 206 amino acid protein (coding region: 275-895 nt) with a molecular weight of 23.8 kDa [Jiang et al., 1995]. Alignment of human *MDA-7* and predicted rat *C49A* protein amino acid sequences showed 58.7% identity globally and 69% identity (82% positives) in the conserved 492 nt coding segment (Fig. 4). Previous studies by Jiang et al. [1995] on human *mda-7* had

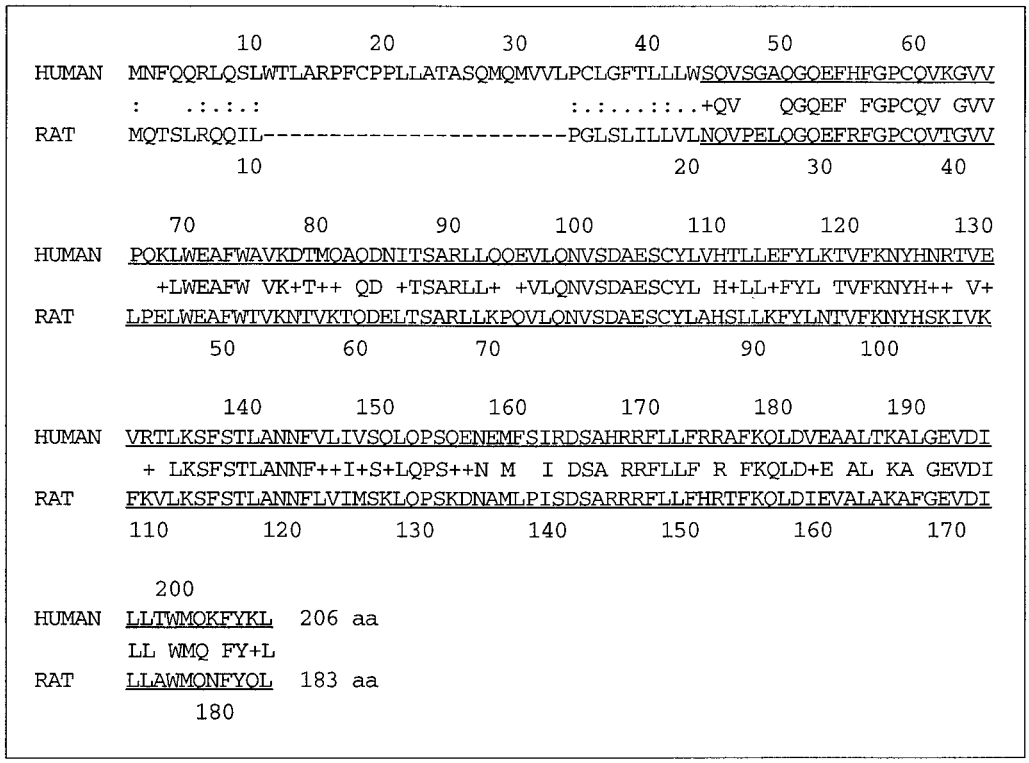

Fig. 4. Global alignment of human *MDA-7* and rat *C49A* amino acid sequences by BLAST at NCBI and ALIGN at EERIE, Nimes, France. There is 58.7% global alignment with 69.3% identity (82% positives) in a 163 amino acid segment (single underline) corresponding to the conserved 492 nt segment.

demonstrated that the *MDA-7* protein was unlikely to be secreted or to be a component of cellular membranes. In addition, neither rat *C49A* nor human *MDA-7* protein sequences showed significant homology to other known proteins, and neither contained any recognized sequence motifs that might suggest a mechanism of action.

C49A Protein Expression at the Wound Edge and Base

Immunohistochemistry using anti-*C49A* antibody was carried out to determine the actual cell type(s) expressing rat *C49A* in 2-day-old wounds. Our results show that rat *C49A* was expressed primarily by spindle-shaped dermal cells at the edge and base of the wound (Fig. 5A–C). Rat *C49A* protein was not observed in 2-day-old wound controls using pre-immune sera (Fig. 5A'–C'), or in nonwounded skin (data not shown).

Human Melanoma and Keratinocytes Contain Identical *Mda-7* Sequences

To determine if there are any differences between the original human *mda-7* isolated from HO-1 melanoma cell lines and other cell types we cloned human *mda-7* from a human papillomavirus (HPV) transformed human keratinocyte cell line, HOK, using specific primer RT-PCR. ALIGN analysis demonstrated that the coding region (nucleotide 275 to 895) for human *mda-7* was identical between HO-1 and HOK cells.

DISCUSSION

Historically, wound repair is divided into three well-characterized, overlapping, histological phases: 1) inflammation, 2) granulation tissue formation, and 3) tissue remodeling [Clark, 1996]. Little, however, is known of the molecular mechanisms behind these histological changes. To more fully elucidate these molecular events, we used DD-PCR in a rat skin wound model to identify significant changes in gene expression that occur during repair. In this study, we demonstrate the identification and isolation of a full-length rat cDNA clone, *c49a*, that exhibits homology to human *mda-7*. We also show the significant up regulation of rat *C49A* protein expression by fibroblast-like cells during wound healing.

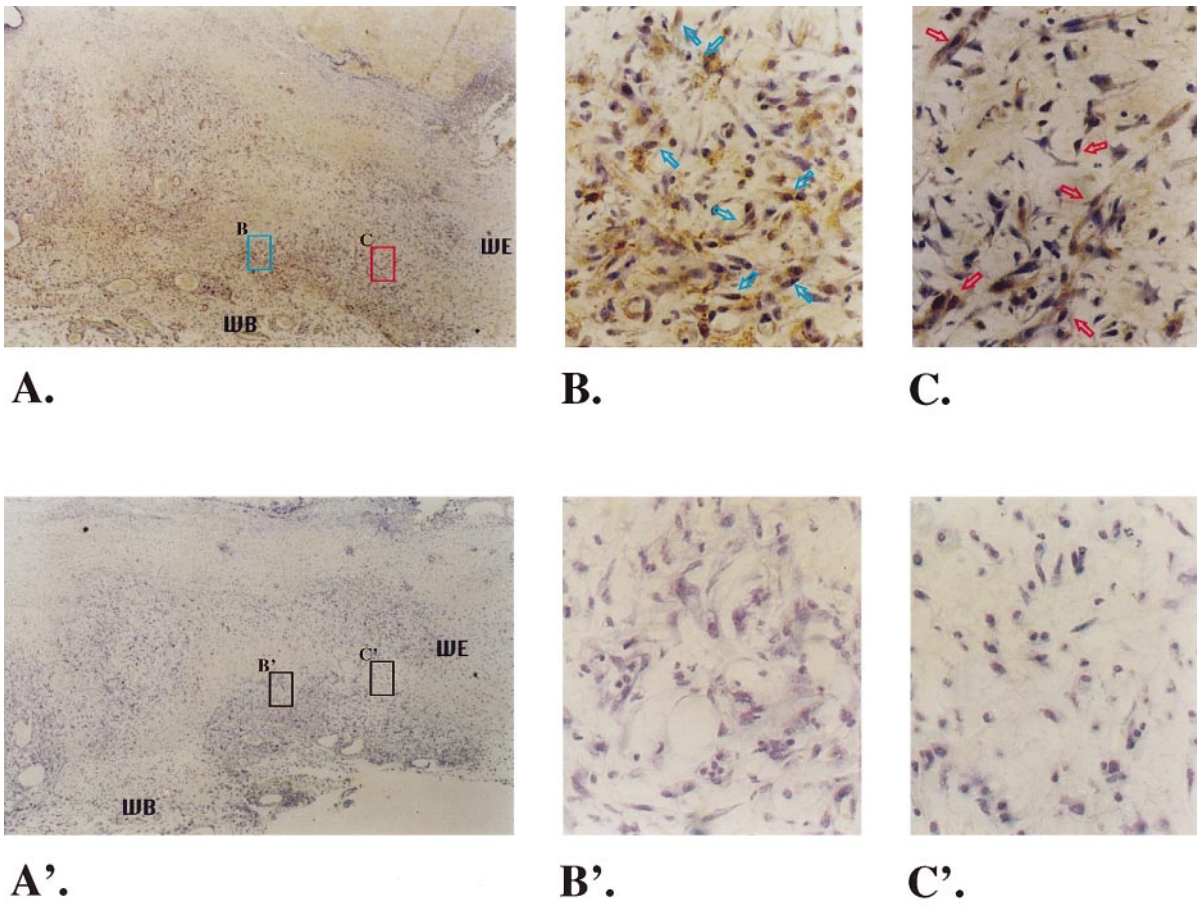

Fig. 5. Immunolocalization of rat *C49A* to wound edge and base. Histologic sections were prepared and immunolocalization performed as described in the Materials and Methods section. Representative sections from 2 day wounds are shown at 40× (**A**) and 400× (**B, C;** original magnification) using 1:500 dilution rabbit anti-*C49A* sera. Controls consisted of pre-immune rabbit serum at 1:500 dilution on 2 day wounds at 40× (**A'**), and 400× (**B', C';** original magnification).

While the exact biologic activity of human *mda-7* and rat *c49a* is unknown, our nucleotide alignment data demonstrating 71.7% homology in the coding region suggest that rat *c49a* is a related molecule to human *mda-7*. Besides similarities in the nucleotide coding region, human *mda-7* and rat *c49a* also exhibit similarities in the non-coding region in the form of mRNA instability elements (Fig. 3). It is noteworthy that these elements are an important mechanism for controlling the rate of mRNA degradation of important transcripts during growth and differentiation [Schiavi et al., 1992]. The mRNA from the immediate early genes such as *c-fos* and *c-myc*, and from cell growth modulators such as granulocyte-monocyte colony stimulating factor and interferon-β all contain within their 3′ untranslated region several AUUUA motifs surrounded by AU-rich sequences [Schiavi et al., 1992; Shaw and Kamen, 1986]. Though both rat *c49a* and human *mda-7* exhibit multiple AUUUA motifs, these motifs do not occur in the context of AU-rich sequences, suggesting that other motifs, possibly in the *c49a/mda-7* coding region, may be responsible for timely *c49a/mda-7* degradation. The high molecular weight *MDA-7* complexing protein identified by Jiang et al. [1995] may also play a role in human *mda-7* stability.

Although the nucleotide alignment data suggest that rat *c49a* and human *mda-7* are homologues, the amino acid alignment data suggests otherwise. Global alignment of rat *c49a* and human *mda-7* amino acids show only 58.7% identity, with an increase to 69% identity (82% positives—note "positives" denotes two homologous amino acids with similar chemical properties) in the highly conserved 492 nt overlap.

The decreased homology seen with amino acid comparisons suggest that rat *c49a* and human *mda-7* may be related molecules rather than true counterparts to each other. Alternatively, there may be large interspecies differences between rat and human versions of *c49a/mda-7*, despite similar zooblot hybridization patterns (data not shown).

In addition to the nucleotide and amino acid sequence data, we have also shown by RT-PCR that nonwounded rat skin express minimal levels of *c49a* (Fig. 2), and that wounded rat dermal cells express markedly elevated levels of *c49a/C49A* before and during the proliferative phase of repair (Figs. 1, 2, and 5). This elevated rat *c49a* expression in dermal wound cells is maintained at least 7–10 days by our RT-PCR data (Fig. 2) and is primarily localized to cells at the edge of the wound base by in situ hybridization (data not shown). Moreover, we have localized *C49A* protein to fibroblast-like cells at the edge and base of the healing wound. In normal, nonwounded skin, fibroblasts are sparsely present and generally quiescent in G_0 phase [Morgan and Pledger, 1992]. By 2 days after acute injury, however, fibroblasts have begun to migrate into the wound and proliferate [Stocum and Karr, 1990]. This suggests that rat *c49a/C49A* expression may be associated with actively dividing cells in the wound; however, more studies involving definitive identification of cell type and demonstration of *c49a* expression with cell proliferation in vitro is required.

Jiang et al. [1996] have demonstrated human *mda-7* to be a ubiquitous growth suppressing gene when transfected into diverse human cancer cell lines. Transfection of human *mda-7* into normal human skin and normal rat embryo fibroblasts cells; however, resulted in quantitatively less growth suppression—albeit still growth suppression [Jiang et al., 1996]. In contrast to the inverse relationship between proliferation and forced *mda-7* expression observed by Jiang et al. [1995, 1996], we have observed a positive association between proliferation and endogenous *mda-7* transcripts in fourth passage, normal human skin fibroblasts grown in 15% fetal bovine serum (FBS) (cell count: 2.18×10^6) as compared to 0% FBS (cell count: 1.12×10^6; data not shown). The direct association between fibroblast proliferation rate and concentration of FBS media is well documented [Temin, 1968, 1970], and our data suggests that increased fibroblast proliferation correlates with increased endogenous *mda-7* expression. This disparity between our findings and Jiang et al.'s results cannot be explained by a mutation in the coding region of their *mda-7* gene isolated from the HO-1 melanoma cell line [Jiang et al., 1995], as we have isolated an identical *mda-7* coding sequence from a HPV transformed keratinocyte cell line, HOK [Park et al., 1991; Shin et al., 1994]. One explanation for the seemingly opposite effects on proliferation between transfected and endogenous *mda-7* expression may be that forced *mda-7* expression produces excessive levels of *mda-7*, with subsequent induction of apoptosis. Acceleration of apoptosis due to overexpression has been described for a number of growth promoting proteins such as c-Myc, c-Myb, B-Myb, cyclin A, and EF2, as well as the E1A and E7 viral oncoproteins [Henriksson and Luscher, 1996]. And while the modulation of apoptosis is not well understood, it may be that growth promoting molecules constitutively activate the apoptotic pathway as a physiological safety mechanism, and that the presence or absence of certain survival factors will then ultimately decide the pathway to cell death or proliferation [Henriksson and Luscher, 1996].

In summary, we have identified a novel rat gene, *c49a*, through DD-PCR applied to an in vivo model of wound repair. We show that rat *c49a* expression is increased in the period preceding and during the onset of cellular proliferation in wound repair. We have also demonstrated that *C49A* protein is expressed primarily by fibroblast-like cells at the healing wound base. We believe that rat *c49a* is a distinct molecule of the *mda-7* family with growth promoting rather than suppressing effects—though the local environment such as cell type, cellular differentiation state, as well as the presence/absence of wound healing cytokines and growth factors may play crucial roles in regulating proliferation. Future studies on *c49a/mda-7*'s precise cellular role, mechanism of action, and expression during development should clarify some of these questions. Finally, these results demonstrate that DD-PCR can provide powerful insight into the biomolecular regulation of wound healing by both the large scale screening of gene expression and by the identification of novel genes associated with repair.

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