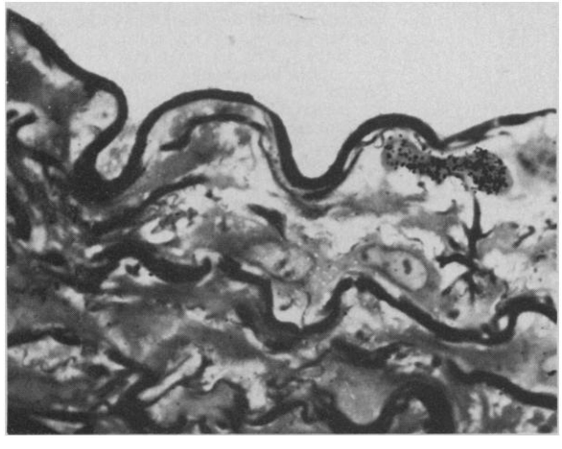
Vascular Smooth Muscle Cell Kinetics: A New Assay for Studying Patterns of Cellular Proliferation in vivo

Abstract. *A new quantitative assay for studying the kinetics of vascular smooth muscle cells in vivo is reported. The assay was used to determine the specific activity of DNA from rabbit aortic smooth muscle cells stimulated to grow by removal of the endothelial layer. The specific activity of the DNA was correlated with the rate of tritiated thymidine incorporation as measured by autoradiography and with the rate of DNA synthesis as estimated by direct measurement of cellular proliferation. Smooth muscle cells exhibit a 24-hour latent period in vivo prior to DNA synthesis; the synthesis peaks at 48 hours and then rapidly declines. The decline in DNA synthesis is not related to endothelial regrowth, and may be of homeostatic significance in limiting luminal stenosis. The assay offers a rapid and reliable alternative to autoradiographic and morphometric techniques for evaluating growth kinetics and growth regulation in vivo.*

Intimal proliferation of smooth muscle cells (SMC's) is central to the development of arteriosclerosis (*1, 2*); characterization of the growth patterns of these cells is therefore essential to an understanding of the disease process. Substantial evidence indicates that intimal proliferation occurs when the vascular endothelium is injured and the underlying SMC's are exposed to bloodborne elements and products of hemostasis (*3*). Tissue culture systems have been used for the study of SMC growth kinetics because these techniques offer the advantage of investigating single cell populations under controlled conditions. However, it was recently suggested that morphologic as well as physiologic alterations occur soon after SMC's are established in culture (*4*). Studies of vascular SMC kinetics in vivo have relied on morphologic and autoradiographic techniques that are time-consuming, difficult to quantitate, and limited to a relatively small sample size. To overcome these difficulties, we have developed a quantitative, reproducible assay for the study of aortic SMC kinetics following intimal injury. The assay offers reliable and rapid analysis of DNA synthesis in SMC's and was used to study the pattern of SMC proliferation in vivo. The data derived from these studies offer new insights into growth and development of intimal lesions.

To prepare the animals for assessment of SMC kinetics, we removed endothelial cells by means of a balloon catheter (*5*). Under light anesthesia, aortas of male New Zealand rabbits were denuded of endothelium by passage of a modified thin-walled Fogarty embolectomy catheter (Edwards Laboratory). The catheter was inserted by way of a femoral arteriotomy and passed to the descending thoracic aorta. The balloon was then inflated with CO_2 to a pressure of 450 mm-Hg and withdrawn to the aortic bifurcation within 10 seconds. Animals were killed by cardiac exsanguination at 1, 8, 16, 24, 32, 40, and 48 hours and 4, 7, 14, 28, and 48 days after balloon injury. Control rabbits without balloon injury but subjected to sham operations were killed at similar times after operation. The number of animals used at every interval sampled ranged from 5 to 15. One hour before each rabbit was killed it was given, per gram of weight, 0.5 μCi of [^{3}H]thymidine (20 mCi/mmole, New England Nuclear) intravenously, followed 30 minutes later by an in-



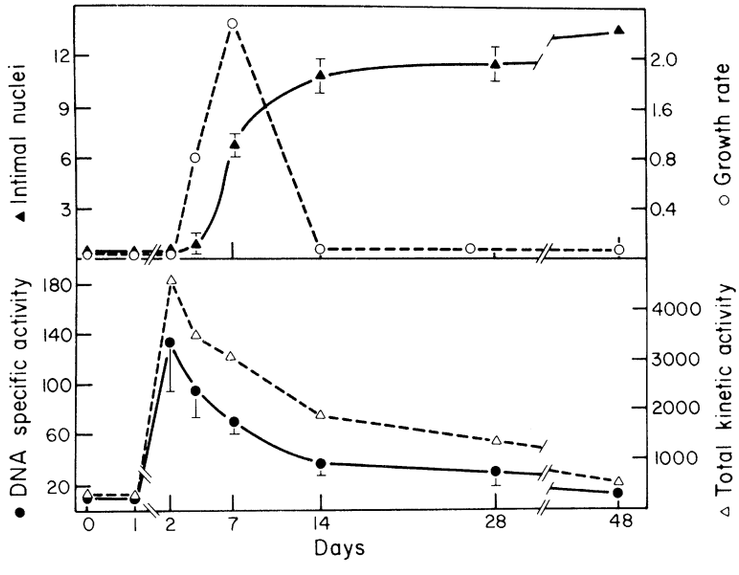


Fig. 1 (left). Autoradiogram of a thoracic aorta 4 days after endothelial injury showing labeled SMC's (×1200). Autoradiography was performed for comparison with the specific activity of DNA from adjoining segments. Three tissue sections in Epon (cut 1 μm thick, 20 μm apart) were prepared from each segment for light microscopy and autoradiography and evaluated as follows. The slides were coated with L-4 (Ilford) emulsion and were incubated for 28 days at 4°C. Nuclei were counted at ×1000 without the observer knowing the slide's origin. The number of intimal and medial nuclei per 0.1 mm of internal elastic lamina was determined with a calibrated eyepiece reticle which was aligned with the internal elastic lamina. The media was divided into inner (first 100 μm from the internal elastic lamina) and outer zones. Intimal nuclei, defined as nuclei above the internal elastica, were counted individually. The average number of medial nuclei per unit length was 34 per 0.1 mm of internal elastic lamina. Numbers of nuclei from intima, inner media, and outer media were pooled. Labeled nuclei were defined as having at least five grains. Correlation of specific activity with total percentage of labeled nuclei per animal was significant ($r = .844, P < .01$). Fig. 2 (right). Specific activity of DNA (expressed as disintegrations per minute per microgram of DNA), total kinetic activity (per 0.1 mm of internal elastic lamina), number of intimal nuclei (per 0.1 mm internal elastic lamina), and intimal growth rate plotted as a function of time. The number of intimal nuclei per 0.1 mm of internal elastic lamina was determined as described in the legend for Fig. 1. Total kinetic activity was calculated by the equation: total kinetic activity = DNA specific activity × (intimal nuclei + 34); 34 is the number of medial nuclei per 0.1 mm of internal elastic lamina. Growth rate is defined as the rate of change of intimal nuclei with time and is computed from the first derivative of the intimal nuclei against the time curve. Each point on the graph represents the mean for each time period and the vertical bars represent 1 standard error.

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travenous injection of 5 ml of Evans blue dye (Harvey Laboratory). This azo dye stains the aortic areas that are devoid of endothelium, thereby confirming endothelial injury in each animal (*6*). Within 1 minute after exsanguination, the thoracic aortic segment from the third to sixth intercostal arteries was removed, immediately frozen, and stored at − 70°C: this segment was used for the determination of the specific activity of DNA. The adjacent sixth to eighth intercostal aortic segment was not frozen, but was immediately immersed in gluteraldehyde and processed for histology and autoradiography (Fig. 1). In addition, duodenal tissues were examined by autoradiography to confirm [^{3}H]thymidine labeling in each animal. Smooth muscle cell DNA synthesis was evaluated both by autoradiography and by measurement of the specific activity of DNA. Comparison of the data obtained by the two methods showed significant correlation (Fig. 1) ($r = .844$, $P < .01$).

The specific activity of DNA was determined as follows. Segments were thawed to 4°C and the adventitias stripped and discarded. The remaining intimal-medial tissues consisted almost exclusively of SMC's (*7*). These tissues were homogenized at 4°C in 0.01*M* tris buffer containing 2 m*M* ethylenediaminetetracetic acid, *p*H 7.5. Pronase (type B, nuclease-free; Calbiochem) (*8*) and sodium dodecyl sulfate (SDS; Sigma) were added to a final concentration of 0.4 percent and 0.5 percent (weight to volume), respectively, and the mixture was incubated at 37°C for 30 minutes. Evans blue was extracted from the digested tissues by adsorbing the dye with heparin-Sepharose 4B beads (*9*) and the DNA content was determined by the method of Burton (*10*). The DNA content in aortas not exposed to Evans blue was similar whether or not the tissue digest was mixed with the beads. Samples were precipitated in 10 percent trichloroacetic acid (*11*), precipitates were trapped on 0.45-μm nitrocellulose filters (Millipore) (*12*), solubilized in 2-methoxyethanol (Eastman), and the disintegrations per minute were determined in a Beckman scintillation counter. Specific activity (disintegrations per minute per microgram of DNA) was calculated and plotted against days after injury (Fig. 2).

Synthesis of DNA, as measured by the specific activity of DNA, remains at base line 24 hours after intimal injury (Fig. 2). Previous studies in vitro have shown that a platelet-derived factor can stimulate SMC's to proliferate (*13*). In the balloon-injured aorta, platelet coverage of the denuded vessel is virtually complete within 10 minutes after injury, and the number of platelet alpha granules is reduced by 97 percent within 40 minutes (*14*). Because alpha granules contain platelet-derived growth factor (PDGF) (*15*), it seems likely that this mitogen is available to SMC shortly after endothelial removal. Thus, the lag in DNA synthesis cannot be attributed to lack of platelet mitogen but may be related to tissue and cellular events preceding DNA synthesis. In vitro this latent period is bypassed.

Peak DNA synthesis occurs by 24 hours after addition of PDGF to quiescent cultured SMC's (*16*). Epidermal cells stimulated by skin incision exhibit a latent period before moving into the S phase of the cell cycle. This latent period is eliminated when epidermal cells are placed in tissue culture after stimulation in vivo (*17*). A latent period of 16 to 18 hours followed by peak DNA synthesis at 24 to 30 hours has been demonstrated in hepatectomy studies in vivo (*18*). It has been suggested that this lag is caused by cellular reorganization that preceeds DNA synthesis. Both RNA polymerase activity and RNA synthesis increase dramatically within hours after hepatectomy as do the activities of other enzymes involved in DNA synthesis (*18*). The characterization of these early cellular events may lead to a further understanding of the mechanisms by which different factors influence SMC growth. Attempts to interfere with the proliferative response could be directed at the latent period. This in vivo model appears suitable for such investigations.

A dramatic increase in DNA synthesis, peaking at 48 hours, follows the latent period (Fig. 2). To quantitate SMC proliferation, intimal nuclei were counted (Fig. 2). Intimal cells grow exponentially between 4 to 7 days doubling three to four times to yield a tenfold increase in cell number. This exponential growth follows the peak in DNA synthesis at day 2 and thus substantiates that [^{3}H]thymidine uptake measurements reflect DNA synthesis. It is clear that such exponential growth, if maintained, would result in occlusion of the vascular lumen. However, intimal proliferation following injury is self-limited. The early 2-day peak of specific activity of DNA declines and rapidly approaches base-line level by day 48 (Fig. 2). To confirm that the decline in specific activity is not secondary to dilution of labeled DNA in an increasing population of intimal cells, we calculated the total kinetic activity per segment (Fig. 2). The decline in total kinetic activity was found to parallel the decline in specific activity. Estimation of intimal growth rate (Fig. 2) shows peak growth rate at day 7 with a 30-fold drop by day 14. The self-limited nature of SMC proliferation in response to injury is of great homeostatic significance in preventing luminal occlusion. The mechanism by which intimal growth is inhibited is not understood. It cannot be attributed to endothelial regrowth, because significant reendothelialization does not occur for several weeks and is incomplete for at least 6 months after deendothelialization (*6*). The decline in the percentage of cells incorporating [^{3}H]thymidine, or the cellular growth fraction, may be associated with either a limited quantity of mitogen or may represent regulation of cellular growth by inhibitory mechanisms. Characterization of these mechanisms can be of significance for understanding vascular SMC kinetics and the pathogenesis of atherosclerosis. In addition, this model can be applied to study the effects of biologic and pharmacologic agents on cellular growth and may prove useful for studying growth regulation in vivo.

ITZHAK D. GOLDBERG
MICHAEL B. STEMERMAN
LOWELL E. SCHNIPPER
BERNARD J. RANSIL
GARY W. CROOKS
ROBERT L. FUHRO

Thrombosis and Hemostasis Unit, Department of Medicine, and Thorndike Laboratory, Beth Israel Hospital at Harvard Medical School, Boston, Massachusetts 02215

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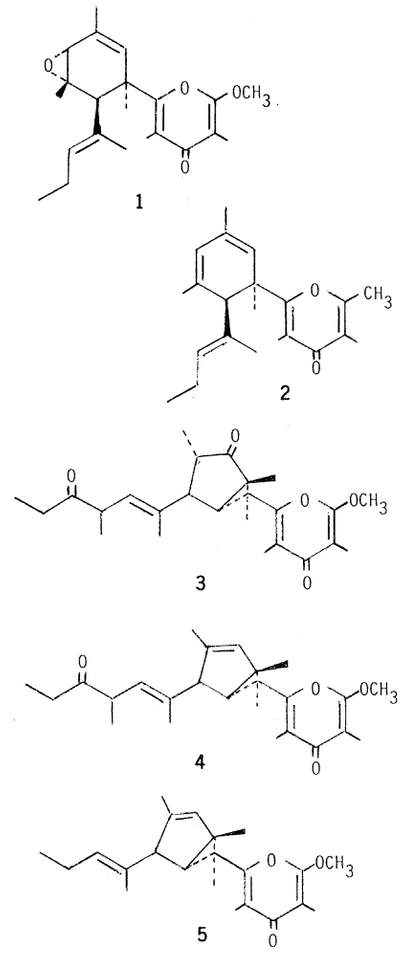
Photosynthetic Marine Mollusks: In vivo ^{14}C Incorporation into Metabolites of the Sacoglossan *Placobranchus ocellatus*

Abstract. *In seawater enriched with carbon-14-labeled sodium hydrogen carbonate the sacoglossan* Placobranchus ocellatus *when exposed to light incorporates carbon-14 at a rate 50-fold of that for animals kept in the dark. 9,10-Deoxytridachione, a secondary metabolite of the mollusk, undergoes a photorearrangement to photodeoxytridachione in vivo.*

The marine mollusk *Placobranchus ocellatus* (Van Hasselt) (phylum Mollusca, class Gastropoda, subclass Opisthobranchia, order Sacoglossa) is one of a unique group of mollusks that assimilate photosynthetically viable chloroplasts from siphonous algae upon which they graze (*1*). We report the results of our ^{14}C-labeling experiments, which indicate that secondary metabolites isolated from *P. ocellatus* are biosynthesized by the mollusk-chloroplast symbiotic pair and that these metabolites are interconverted through an unprecedented in vivo photoisomerization. These biosynthetic experiments are a result of our recent research into the constituents of the sacoglossans *Tridachiella diomedea* and *Tridachia crispata* (*2–4*).

Trench *et al.* (*5*) had previously investigated the primary metabolism of *T. diomedea*, *T. crispata* and *P. ocellatus* by radioactive isotope labeling techniques. They demonstrated that organic carbon fixed during photosynthesis is transmitted to chloroplast-free animal tissue. The greatest concentration of ^{14}C occurs in the digestive diverticula and the pedal mucus gland (*1*). Chromatographic analysis indicated the presence of labeled carbon in a wide variety of primary metabolites, including lipids, amino acids, and primarily mono- and oligosaccharides.

During research into the secondary metabolites of sacoglossan mollusks, we isolated tridachione (**1**) and 9,10-deoxytridachione (**2**) from the ether-soluble oil of *T. diomedea* collected in the Gulf of California (*2*), as well as crispatone (**3**) and crispatene (**4**) from *T. crispata* collected in the Caribbean (*3*). The structural relation between these two carbon skeletons was demonstrated by the in vitro photochemical isomerization of **2** to photodeoxytridachione (**5**) (*4*). The interconversion in vitro of these skeletons suggested to us that this transformation occurs in vivo and represents an unprecedented biosynthetic step. Furthermore, the well-documented photosynthetic capability of the mollusk-chloroplast symbiotic pair suggested that these secondary metabolites are biosynthesized in situ by the symbiotic pair.



To confirm our hypothesis, we required a sacoglossan that contained both carbon skeletons, thus facilitating labeling experiments. We investigated *P. ocellatus*, which we collected at Ala Moana beach, Oahu, Hawaii. Silica-gel chromatography of the acetone extract of *P. ocellatus* yielded a single ultraviolet-absorbing band (eluant, a mixture of ether and hexane, 50 : 50). Reversed phase chromatography with an octadecylsilane-coated support (eluant, a mixture of methanol and water, 80 : 20) resolved the band into three isomeric components. The two major components were readily identified by spectral analysis (^1H and ^{13}C nuclear magnetic resonance) and comparison with authentic samples as 9,10-deoxytridachione (**2**) and photodeoxytridachione (**5**). The minor component (**X**) appears to be an epimer of **2**, but the amount of available material was too small for complete characterization.

We designed our initial labeling experiments to show that these metabolites are produced in situ under photosynthesis conditions. Specimens of *P. ocellatus* were placed in aerated Millipore-filtered seawater (4 ml per animal) enriched with ^{14}C-labeled sodium hydrogen carbonate at 20 μCi/ml. Four animals ("light" animals) were incubated in sunlight at 25°C for 8 hours, then transferred to the dark for an additional 18 hours. Two animals ("dark" controls) were maintained in the dark for 26 hours. The four light animals were killed and extracted with methanol. The combined extracts were partitioned between ether and 20 percent aqueous NaCl. The resulting ether layer was dried over $MgSO_4$ and evaporated, yielding an oil. The dark animals were treated similarly. The light animals yielded 12 mg of oil, the dark control yielded 4 mg. Each extract was diluted with methanol to 1 ml; a 10-μl portion of the light animal extract had 18,020 count/min above background, while a 30-μl portion of the dark control extract showed 351 count/min, which is less than 2 percent incorporation compared with the light animals. All samples were counted to a 95 percent confidence level (*6*). Identical portions were chromatographed on an Eastman Kodak silica gel thin-layer chromatograph (TLC) sheet (elution with diethyl ether) and the individual bands were assayed (Table 1). Material

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