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48. We thank R. Sweet and M. Rosenberg for *ras* protein, J. Wang for the antibody to phosphotyrosine, B. Bui for secretarial assistance, and C. Queen and J. Tso for comments on the manuscript. This work was supported by grants from the National Institutes of Health (AI 21298) and March of Dimes Birth Defects Foundation (1-915) to L.J.K. and by the National Institutes of Health (AM 34926 and AM 01393) and an American Cancer Society Institutional Research Grant (IN 32Y and IN 32Z) to R.A.R. L.J.K. is an American Cancer Society Junior Faculty Research Awardee.

23 September 1986; 6 February 1987

Chemical Identification of a Tumor-Derived Angiogenic Factor

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Neoplasms produce substances that induce blood vessel formation (angiogenesis). Fractions from ethanol extracts of the Walker 256 carcinoma were isolated by silica column chromatography and C_{18} reversed-phase high-performance liquid chromatography. Two of the isolated fractions induced neovascularization when tested in the rabbit corneal micropocket assay. One of the fractions was identified as nicotinamide by desorption–electron impact mass spectrometry, nuclear magnetic resonance spectroscopy, and gas chromatography–mass spectrometry. The second active fraction contained nicotinamide as part of a more complex, as yet unidentified, molecular arrangement. Microgram quantities of commercial nicotinamide induced neovascularization in the corneal micropocket assay and in the chick chorioallantoic membrane assay.

ANGIOGENESIS IS THE PROCESS whereby blood vessels proliferate. The process is a normal part of embryonic development, wound healing, and limb and organ regeneration. It plays a facilitative role in disease states such as cancer (*1*) and numerous retinopathies (*2*). Some neoplasms release factors that induce angiogenesis in experimental models (*1*). Even normal tissue at risk for malignancy (preneoplastic) bears an increased capacity to induce angiogenesis (*3*). Well-characterized proteinaceous substances that are angiogenic include basic fibroblast growth factor (FGF) (*4*), α-transforming growth factor (*5*), and angiogenin (*6*). No low molecular weight, organic, tumor-derived angiogenic substances have yet been identified.

The Walker 256 carcinoma has served as a classic source of angiogenic factors (*1, 7*). Low molecular weight extracts with light absorbance maxima at 260 nm were observed by Vallee *et al.* (*7*) and by Fenselau *et al.* (*8*). The extract described by Fenselau *et al.* (*8*) was partially purified by silica gel chromatography. It stimulated blood vessel proliferation in two models of angiogenesis in vivo, the rabbit corneal micropocket assay and the chick chorioallantoic membrane. It also stimulated endothelial cell growth in culture.

We have isolated two active moieties from similar extracts. One was identified as nicotinamide, a vitamin and a component of the ubiquitous cofactors nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The other active component was a more complex molecular arrangement that contained nicotinamide (or derivatives). Walker 256 carcinoma cell homogenates were extracted, and the extracts were run on sequential silica gel columns (*8, 9*). The capacity of fractions to stimulate endothelial cell proliferation was determined as detailed elsewhere (*10*). The active eluate from the silica column was further fractionated by reversed-phase high-performance liquid chromatography (HPLC). Three major and numerous minor peaks were obtained (*11*). The major peaks were examined for angiogenic activity in the rabbit corneal micropocket assay (*12*). This assay involves surgically implanting test material contained in a vinyl polymer pellet.

Figure 1 compares the rates of vessel growth. Material from peak 3 (P3) showed strong activity in the assay. Evidence for the structure of P3 was first derived from a desorption–electron impact mass spectrum (*13*). The spectrum was identical to that of commercial nicotinamide (Fig. 2). Comparison of HPLC retention times in two systems (*11*), ultraviolet (UV) absorbance spectra (*11*), nuclear magnetic resonance (NMR) spectrometry (*14*), and gas chromatography–mass spectrometry (*15*) provided additional agreement. All data were identical. No other organic constituents were apparent in the tumor-derived P3 material. An equivalent amount of commercial nicotinamide (40 μg per pellet) was also active in the micropocket assay, but vessel outgrowth commenced later (Fig. 1). A separate trial of nicotinamide showed activity at 20 μg per pellet. Microgram quantities of

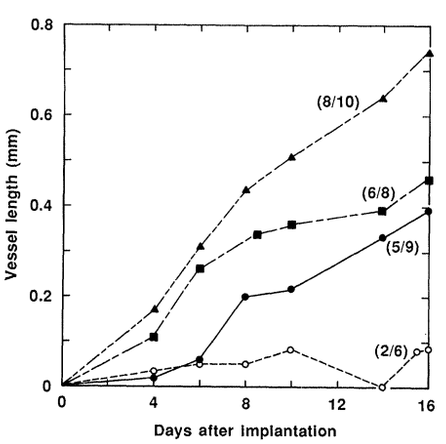

Fig. 1. Rate of vessel growth. Angiogenesis was assessed by the corneal micropocket assay in rabbits as described (*12*). Eight A_{260} units of test material were incorporated into ten 1.5-mm^3 Elvax (a vinyl polymer) pellets. Each pellet was implanted in a cornea, 1 mm from the limbus. The number and length of new vessels growing from the limbus to the pellet were determined three times weekly for 2 weeks. Points show the average vessel length. The standard error of the mean for each point was approximately 30% of its value. Parenthetical numbers indicate the number of implants eliciting any angiogenic response relative to the number evaluated. Inflamed corneas were not included in the tabulation. Nicotinamide-induced angiogenesis was noted for its lack of inflammation as assessed by stereomicroscopy. ▲, P3; ■, P2; ●, commercial nicotinamide; ○, P1.

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nicotinamide also produced an angiogenic response in the chick chorioallantoic membrane (*16*).

A second peak (P2) was also active in the micropocket assay (Fig. 1). This peak appeared to be a complex mixture composed of nicotinamide or its derivatives, sugar, and unknown substances. The desorption–electron impact mass spectrum of this material exhibited intense ions corresponding to nicotinamide (*13*). Nicotinamide was probably released by pyrolysis. A gas chromatography–mass spectrometry experiment with the trimethylsilyl derivative of P2 gave a single large peak (*15*). The mass spectrum had several series of ions corresponding to a trimethylsilylated sugar and phosphate. The NMR spectrum of P2 indicated the presence of aromatic protons, H1 of a sugar with virtual coupling to H3, other sugar peaks, some long-chain aliphatic protons, and silicon grease. The spectrum did not correspond to that of NAD or NADP. No further characterization was attempted. The third major peak (P1) isolated from tumor extract lacked angiogenic activity. It was not characterized.

Since the angiogenic response of tumor-derived nicotinamide and commercial nicotinamide were not identical, the release rates from pellets that had similar formulations were compared by in vitro studies (*17*). The tumor-derived samples contained potassium phosphate that was acquired from the HPLC fractionation; although attempts were made to remove the salt, 30 μg of phosphate were contained in the tumor-derived formulations. No salt was incorporated in the commercial nicotinamide pellets. Exponential release rates similar to those reported for other substances were observed (*18*). However, salt enhanced the initial burst rate. Twice as much nicotinamide was released during the first 48 hours (14 μg versus 6 μg), after which the release rates of both groups were approximately 0.5 μg/day. Thus, the lead times for the angiogenic responses of the tumor-derived materials may be related to their initial, enhanced rates of release.

Nicotinamide is a component of mammalian cell medium formulations. In vitro studies confirmed a requirement for nicotinamide or nicotinate for the growth and maintenance of cultured endothelial cells. The concentration of nicotinamide is 0.2 μ*M* in medium 199, which was used with 2% dialyzed fetal bovine serum in our routine endothelial cell proliferation assay. Concentrations in excess of those used in our standard conditions did not enhance the proliferation rate, and none of the fractionated peaks demonstrated proliferation activity in our standard assay conditions. More

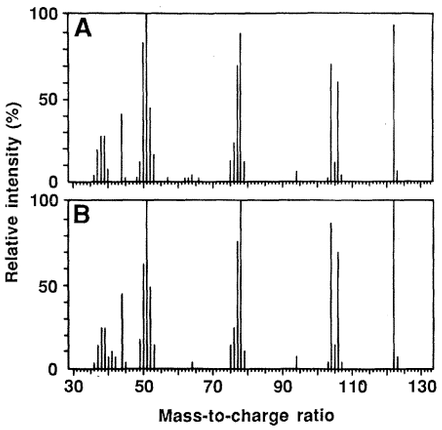

Fig. 2. Electron impact mass spectrometry. (**A**) The electron impact mass spectrum of P3. (**B**) The electron impact mass spectrum of commercial nicotinamide. Relative intensity is measured as a percentage of the most intense peak.

crude fractions of tumor extract were proliferative (*8–10*). Those fractions may have supplied a nutrient mixture that enhanced proliferation. Alternatively, they may have contained a mitogen that was lost during fractionation. Thus, the possibility of other endothelial cell chemoattractants or mitogens is not challenged by our finding.

The concentrations required for angiogenesis appear high relative to proteinaceous growth factors, but the tissue concentrations of nicotinamide are also likely to be much higher. The concentrations of nicotinamide found in normal rat tissues are on the order of tens of micromoles per kilogram of wet weight (*19*), whereas the concentration of basic FGF in bovine brain is nanomoles per kilogram of wet weight (*20*). The molar ratio of the amounts required to stimulate angiogenesis in the rabbit cornea relative to the amounts found in normal tissues is approximately equal.

The mechanism by which nicotinamide is angiogenic is unknown. It has been suggested that basic FGF (*4*) and ischemic metabolites such as lactic acid (*1*) are substances found in normal and tumor tissues that may be expressed by tumors to induce angiogenesis. Nicotinamide may be such a factor. The hydrolysis of NAD and NADP in ischemic myocardium appears to be an early event (*21*) that precedes the histologic detection of necrosis (*22*). The Walker 256 carcinoma has an enhanced capacity to metabolize nicotinamide (*19*). The mechanism may be nutritious. Increased levels of nicotinamide may be required for growth and differentiation. The two enzymes known to use NAD as a substrate, NAD glycohydrolase (*23*) and poly[adenosine diphosphate (ADP)–ribose] synthetase (*24*), are induced in embryogenesis. Poly(ADP-ribose) synthetase is strongly implicated in differentiation (*25*).

The mechanism may be related to that of the vasodilating agents xanthinol nicotinate and dipyridamole. Chronic treatment of rats with dipyridamole induces proliferation of capillary endothelial cells (*26*), and chronic treatment of humans with xanthinol nicotinate, a mixture of theophylline and nicotinic acid, was found to enrich the microvasculature (*27*). Regardless of the mechanism, one practical application of an angiogenic activity is to expedite wound healing. Sodium nicotinate, a related structure that has an acute vasodilating property, has been found to enhance experimental skin flap healing in combination with oxygen (*28*). Xanthinol nicotinate enhanced healing in rat skin flaps and lesions (*29*) and in human split grafts (*30*).

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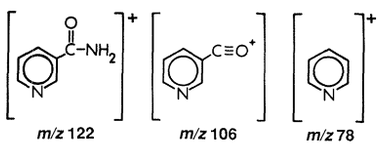
11. Crude tumor extract from 10^{11} Walker 256 carcinoma cells was prepared and purified as described (*8, 9*). The active eluant (by endothelial cell proliferation) from the second silica column was dried by rotoevaporation and solubilized in methanol. Fifteen units of absorbance at 260 nm (A_{260}) were applied to a semipreparative C_{18} μBondapak reversed-phase HPLC column (Waters Associates) and eluted in 10 m*M* potassium phosphate, *p*H 7.0, with a 10-minute linear gradient of 0 to 2% methanol. The absorbance at 260 nm was monitored. Discrete major peaks eluted at 8, 12, and 20 minutes (called P1, P2, and P3, respectively). Similar peaks from five identical runs were pooled, freeze-dried, and desalted by being run through the same column in water with a gradient of 0 to 2% methanol or through C_{18} cartridges (Sep-Pak, Waters Associates). Each major peak was homogeneous by both reversed-phase and silica HPLC. Each had UV absorbance peaks near 260 nm. They were present in the active eluant (by endothelial cell proliferation) in approximately equal amounts (by A_{260}). Peak 3 and commercial nicotinamide migrated identically. Maximum UV absorption of both these peaks was 261 nm, and their UV absorbance spectra were identical. The yield of P3 (nicotinamide) was 400 μg. The fractionation procedures were repeated on two additional lots from 10^{11} cells with similar results. The percentage of recovery was determined by adding [*carbonyl*-^{14}C]nicotinamide to a fourth lot of tumor homogenate and monitoring reactivity and A_{260} throughout the purification. The percentage of recovery was 16% in the ethanol extract, 9% in the first silica column eluant, and 2% in P3. The relative contribution of nicotinamide to the in vivo activity of tumor homogenate was not determined. The activities of ethanol extract and a partially purified fraction have been measured (*8, 9, 12*). On the basis of our recoveries and the reproducibility of fractionations, angiogenic pellets composed of ethanol extract and the first silica column eluant (*12*) were estimated to have contained approximately 0.3 and 1 μg of nicotinamide, respectively. Metabolites, cofactors, and related structures (such as P2) would also have been present and may have contributed to the activities of these crude fractions.

12. The corneal micropocket assay was performed in rabbits by G. Lutty and R. Mello, of Johns Hopkins University School of Medicine, by methods similar to those described by G. A. Fournier, G. A. Lutty, S. Watt, A. Fenselau, and A. Patz [*Invest. Ophthalmol. Visual Sci.* **21**, 351 (1981)].
13. Methanol solutions of P2, P3, or commercial nicotinamide were analyzed with a Nermag R 10-10 quadrupole mass spectrometer; a 60-μm tungsten wire was used for the desorption surface. The three largest ions observed, mass-to-charge ratio (*m/z*) 122, 106, and 78, are suggested in the diagram below. For the methodology, see B. Soltmann, C. C. Sweeley, and J. F. Holland [*Anal. Chem.* **49**, 1164 (1977)] and D. F. Hunt, J. Shabanowitz, F. K. Botz, and D. A. Brent (*ibid.*, p. 1160).



14. The NMR spectrum data are as follows: δ 9.03 (1H, dd, $J_{2,4}$ = 2.1, $J_{2,5}$ = 0.6, H2); δ 8.70 (1H, dd, $J_{5,6}$ = 4.8, $J_{4,6}$ = 1.7, H6); δ 8.22 (1H, ddd, $J_{4,5}$ = 7.9, H4); and δ 7.50 (1H, ddd, H5).
15. A Durabond DB-1 (J and W Scientific) capillary column was programmed from 100° to 270°C at 10° per minute. Electron impact was the mode of ionization. The mass spectrometer was a Nermag R 10-10 quadrupole.
16. M. N. Ellis, unpublished results.
17. Elvax 40 "sandwich" pellets similar to those used in Fig. 1 were made, except that they incorporated 0.1 μCi of [*carbonyl*-^{14}C]nicotinamide with and without potassium phosphate. Pellets were placed in 0.25 ml of Hanks buffer. The solution was replaced at 1, 2, 4, 8, 24, 48, 72, 96, 192, 288, and 384 hours. Release rates were related to the recovery of radioactivity. Fifteen percent of the nicotinamide was released in the first 48 hours as compared to 36% in the group of pellets containing the salt. The release rates were not significantly different over the next 12 days.
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24 September 1986; accepted 2 March 1987

Efficient Packaging of Readthrough RNA in ALV: Implications for Oncogene Transduction

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Readthrough viral transcripts are present at relatively high levels in cells infected with avian leukosis virus. It has been proposed that they can function as intermediates in the transduction of proto-oncogenes by retroviruses. It is shown here, by the analysis of viruses containing a mutation in the AAUAAA polyadenylation signal, that readthrough RNAs have the requisite properties to function as transduction intermediates: (i) readthrough RNAs were polyadenylated and packaged as efficiently as normal viral RNA, (ii) RNAs nearly 11.2 kilobases (3.5 kilobases larger than wild-type avian leukosis virus genomes) were present in virions of the mutant virus, and (iii) virus particles containing both readthrough and normal genomes were most likely infectious.

INTEGRATED PROVIRAL DNA OF RETROviruses is flanked by long terminal repeats (LTRs) that contain transcriptional regulatory signals recognized by the host cell. Although identical in sequence, the LTRs are distinct in function. Viral transcription is initiated within the 5′ (upstream) LTR; cleavage and polyadenylation of viral transcripts occur within RNA derived from the 3′ (downstream) LTR. Transcripts initiated within the 3′ LTR (downstream transcripts) are normally undetectable (*1*).

Integration of proviral DNA in the vicinity of cellular proto-oncogenes can cause malignant disease in the host, due to the effects of viral transcriptional signals on the expression of adjacent cellular genes. In avian leukosis virus (ALV)–induced erythroblastosis, the expression of the proto-oncogene c-*erb*B can be activated by readthrough transcripts, that is, viral RNAs that escape cleavage and polyadenylation at the normal site and thus extend into the downstream c-*erb*B gene (*2*). In ALV-induced lymphoma, expression of the c-*myc* gene is usually activated by viral transcripts initiated within the 3′ LTR, that is, downstream RNAs (*3*). In addition, new ALV-related viruses that contain oncogenes are occasionally recovered from animals with ALV-induced malignancies (*4, 5*).

Proviral integration within or adjoining a proto-oncogene is thought to be an essential step in the acquisition of oncogenes by retroviruses. A multistep model has been proposed for oncogene capture as follows (*6*): (i) integration of proviral DNA within or upstream of a proto-oncogene, in the same transcriptional orientation; (ii) deletion of the 3′ proviral DNA to fuse the proto-oncogene to the viral transcriptional unit; (iii) transcription of the deleted provirus to produce an RNA containing both viral and proto-oncogene sequences; (iv) packaging of the chimeric transcripts and normal viral genomes into virus particles; and (v) use of both molecules as templates during reverse transcription, that is, template switching (*7*), resulting in the formation of a new provirus containing the oncogene. Alternatively, oncogene transduction could be accomplished entirely at the RNA level, without a chromosomal deletion, by the generation and packaging into virions of readthrough transcripts that contain the proto-oncogene (*8*). This scheme requires (i) that readthrough transcripts be produced and efficiently packaged into infectious virus particles and (ii) that virus particles accommodate readthrough RNAs of adequate size. In previous work we found readthrough RNAs at high levels in infected cells, amounting to 15% of the total viral RNA (*1*). In this report we show efficient incorporation of readthrough RNAs into infectious virus. Furthermore, virions can accommodate RNAs nearly 11.2 kb in size, 3.5 kb longer than the genome RNA of ALV.

To characterize the biological effects of readthrough transcripts, we found it desirable to increase their abundance. Thus, we introduced a point mutation into the polyadenylation [poly(A)] signal of a DNA clone of Rous sarcoma virus (RSV) by means of oligonucleotide-directed mutagenesis (Fig. 1). Infectious virus was recovered after transfection of turkey embryo fibroblasts (TEF) with the mutant and wild-type DNAs, and was used to infect fresh TEF

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