Telomerase Activity in Melanocytic Lesions

A Potential Marker of Tumor Biology

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Telomerase activation, being a cardinal requirement for immortalization, is a crucial step in the development of malignancy. With a view toward diagnostic and biological aspects in melanocytic neoplasia, we investigated the relative levels of telomerase activity in 72 nevi and 16 malignant melanomas by means of a modified telomeric repeat amplification protocol (TRAP) assay, including an internal amplification standard. We further compared telomerase activity with the expression of two different proliferation-specific proteins, Ki-67 and repp86, a protein expressed exclusively in the cell cycle phases S, G2, and M. Telomerase activity was associated with the overall growth fraction (Ki-67) but showed a closer correlation with the expression of repp86. Both telomerase activity and proliferation indices discriminated clearly between malignant melanomas and nevi, but not between common and dysplastic nevi. Nonetheless, a portion of nevi exhibited markedly elevated telomerase activity levels without proportionally increased proliferation. This was independent of discernible morphological changes. Clinicopathological correlations showed an association between high telomerase activity and early metastatic spread in melanomas, linking telomerase to tumor biology. Our results provide arguments in favor of an occasional progression from nevi to melanomas and imply that proliferation measurements in combination with telomerase assays may help to elicit early malignant transformation that is undetectable by conventional morphology. (*Am J Pathol* 2000, 156:1425–1432)

The proliferative potential of normal cells is limited by the gradual loss of hexameric guanine-rich tandem nucleotide repeats at the chromosomal ends (termed *telomeres*).

^{1,2} Telomere shortening occurs with each cell division because of the inability of DNA polymerases to replicate the ends of linear DNA.^{3,4} In this way, telomeres constitute a “mitotic clock,” the critical reduction of the telomere length, leading to the induction of replicative senescence and eventual cell death.^{5,6} Malignant cells, on the other hand, have the capacity to proliferate indefinitely and therefore must be able to compensate for telomere attrition. The main mechanism of this effect is carried out by the ribonucleoprotein enzyme telomerase, which synthesizes telomeric DNA onto chromosomal ends.^{7,8} Accordingly, in contrast to the majority of mammalian somatic cells, most immortal cells examined to date exhibit significant levels of telomerase activity⁹ and show no net loss of telomere length during proliferation.^{10,11} Telomerase activity has also been found in the majority of common human cancers^{12,13} and in some instances is already detectable at premalignant or *in situ* stages.^{14–19}

It is therefore tempting to use the telomerase assay as a complementary method to distinguish between benign and malignant tumors. Indeed, comparisons of telomerase activity with cytological examinations and other clinicopathological parameters have provided arguments in favor of this idea.^{20,21} One must nevertheless consider that telomerase activity is physiologically present in germline cells and the precursors of spermatogenesis,^{22,23} and at lower levels in regenerative tissues, such as the gastrointestinal mucosa,²⁴ cyclic endometrium,^{25,26} germinal centers of lymph nodes and progenitors of hematopoietic lineage,^{27,28} stem cells of the epidermis,^{29,30} and hair follicles.³¹ Moreover, telomerase can be induced by UV irradiation and inflammatory changes.^{32–34} Hence, telomerase cannot be regarded as a marker of malignancy. However, a careful comparison of the relative activity levels in different tumors of common histogenesis may yield specific diagnostic information.

With this in view, we assayed telomerase activity in a variety of melanocytic lesions comprising simple lentigos, different types of nevi including dysplastic (atypical) nevi, and primary malignant melanomas at various stages of

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Figure 1. a: Typical dysplastic nevus with relatively high telomerase activity (520 CE; see Figure 2). There is architectural disorder, lentiginous melanocytic hyperplasia, and concentric eosinophilic hyperplasia, and patchy perivascular lymphocytic infiltrates are present in the papillary dermis (H&E stain; original magnification, $\times 140$). **b:** At higher magnification ($\times 350$), random nuclear atypia of melanocytes is noted.

tumor progression. Telomerase activity was further compared with the proliferation rate of the tumors cells assessed immunohistochemically by means of two monoclonal antibodies: Ki-S11,^{35,36} which is directed to a formalin-resistant epitope of the Ki-67 protein defining the so-called growth fraction, and Ki-S2.^{37,38} The latter recognizes a proliferation-specific protein expressed from the onset of S phase until mitotic cytokinesis,³⁹ which has been renamed repp86, according to its theoretical molecular mass. We will show that telomerase activity is low in benign melanocytic proliferations and in most dysplastic nevi and high in malignant melanomas, in which it correlates with cellular proliferation. However, in rare cases of dysplastic nevi, unexpectedly high levels of telomerase activity were observed, which were associated with a higher relative percentage of cells in S through M phases. The activation of telomerase in some nevi might indicate a first step in the acquisition of malignant potential.

Materials and Methods

The material consisted of excisional biopsies of 72 benign melanocytic lesions and 16 malignant melanomas.

Fifty-one nevi presented with atypical gross features, i.e., irregular contours and ill-defined borders, variegated color, and often a diameter greater than 5 mm. Histologically, common nevi and malignant melanomas were diagnosed on the basis of standard diagnostic criteria.^{40,41} For the diagnosis of dysplastic nevi, the criteria defined by Elder and associates^{42,43} were applied. The diagnosis of dysplastic nevus was histologically confirmed in 35 of these cases (Figure 1). All cases diagnosed clinically as malignant melanomas also fulfilled the histological criteria for this diagnosis. Tumor and patient characteristics are detailed in Table 1. Five samples of normal skin taken from UV protected areas served as controls in the telomerase assay. Immediately after excision, the tissue specimens were divided in half. One half was snap-frozen in liquid nitrogen and subsequently stored at -80°C ; the other half was fixed in 10% formalin for 20 hours and embedded in paraffin. From the paraffin blocks, serial sections were cut for conventional histology and immunohistochemistry. The hematoxylin and eosin (H&E)-stained sections were reviewed for diagnosis by two independent dermatopathologists (S. J. and P. R.). Clinical follow-up data were obtained from the referring physicians.

Table 1. Tumor and Patient Characteristics

Diagnosis	n	Age (years; median/[range])	M:F
Common nevi	37	24 [12–64]	19:18
Simple lentigo	2	35/45	1:1
Junctional nevus	3	32 [29–35]	1:2
Compound nevus	22	21 [12–64]	13:9
Dermal nevus	10	24 [18–48]	4:6
Dysplastic nevi	35	30 [14–48]	18:17
Junctional	14	29 [16–38]	6:8
Compound	21	31 [14–48]	12:9
Malignant melanomas	16	48 [29–68]	7:9
Melanoma <i>in situ</i>	1	51	0:1
Invasive melanoma < 1.5 mm	8	48 [29–63]	4:3
Invasive melanoma > 1.5 mm	3	53 [44–68]	2:4
Metastatic melanoma	2	38/64	1:1

Telomerase Assay

Samples of fresh-frozen lesional tissue with a maximum size of 0.6 × 0.2 × 0.2 cm were lysed in 100 μ l of freshly prepared CHAPS buffer (10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, 0.5% 3-[(3-cholaunidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 1 mmol/L 4-(2-aminoethylbenzenesulfonyl fluoride (AEBSF) (Sigma, Hamburg, Germany), 1 mmol/L dithiothreitol (DTT), and 1 μ l (100 U) RNase inhibitor (RNasin) (Promega, Hamburg, Germany) for 30 minutes on ice (all other reagents were purchased from Merck (Darmstadt, Germany) unless stated otherwise). After centrifugation (16,000 × g) for 20 minutes at 4°C, the supernatants were divided into 20- μ l aliquots for further use and storage. Protein concentrations were measured with use of the Bradford assay (BioRad, Munich, Germany). The telomeric repeat amplification protocol (TRAP) assay^{44,45} was performed in two steps. In the elongation step, 5 μ g protein was incubated in a final volume of 50 μ l of assay buffer containing 20 mmol/L Tris-HCl (pH 8.3), 63 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.008% Tween, deoxynucleoside triphosphates (Qiagen, Hilden, Germany; final concentration of 0.2 mmol/L each), and 10 pmol N,N,N',N'-tetramethyl-6-carboxyfluorescein (TAMRA)-labeled forward primer TS (Eurogentec, Cologne, Germany) for 25 minutes at 25°C. The samples were extracted once with phenol-chloroform and once with chloroform alone, followed by ethanol precipitation. The pellets were then redissolved in 50 μ l of assay buffer (as above) supplemented with 10 pmol fluorescein-labeled reverse primer CX-ext (Eurogentec), 2.5 U Taq-polymerase (Amplitaq Gold), and 0.01 attomol of an internal amplification standard (ITAS) prepared as described.⁴⁶ This internal standard is necessary for reliable quantification of telomerase activity in different tissue extracts because the polymerase chain reaction (PCR) amplification of telomerase products may be influenced by variable protein concentrations and the presence of inhibitors. Samples were transferred to a thermocycler for 35 cycles of PCR amplification (30 seconds at 95°C, 30 seconds at 50°C, and 30 seconds at 72°C) and subsequently analyzed by capillary electrophoresis (ABIprism 310; Perkin-Elmer, Foster City, CA). The number of cycles was optimized with use

of the cell line L428.⁴⁵ Integrated values were added up for all telomerase products containing five (one repeat beyond the primer dimer size) to 9 telomeric hexamer repeats and calibrated by division by the value obtained for ITAS. Different concentrations (500, 250, 100, and 50 cells/50- μ l assay, corresponding to 200, 100, 40, and 20 ng protein/assay, respectively) of the highly telomerase-active cell line L428 served as positive controls. These were analyzed analogously and used to generate a calibration curve. In this way, telomerase activity of the samples under investigation can be expressed in terms of "cell equivalents" (CE) corresponding to multiples of the activity of one L428 cell. All telomerase assays were done at least in triplicate. When the ITAS product became undetectable in the presence of exceedingly high relative telomerase levels, dilution steps were performed until quantitation became possible.

Immunohistochemistry

Immunostaining procedures were as previously described.^{35,37} Briefly, 4- μ m-thick sections were cut from the paraffin blocks and routinely processed. Endogenous peroxidase activity was blocked by 3% (v/v) hydrogen peroxide in methanol for 5 minutes. Antigen retrieval was effected by boiling the slides immersed in 0.01 mol citric acid (pH 6.0) for 2.5 minutes in a pressure cooker.⁴⁷ The primary antibodies were then incubated on the sections for 30 minutes at room temperature. Antibodies Ki-S2 (repp86) and Ki-S11 (Ki-67) (both from our laboratory) were used undiluted as lyophilized cell culture supernatants reconstituted with 5 ml H₂O. The immunoreactions were enhanced by means of the streptavidin-biotin-peroxidase technique with the use of a rabbit anti-mouse antibody, followed by counterstaining with Mayer's hematoxylin. The total tumor surface on at least one section was evaluated, and the quantity of positive nuclei was expressed as a percentage of the total tumor cell count.

Immunostains and telomerase assays were evaluated without knowledge of clinical data.

Statistics

The CSS statistical software was used for all analyses. Continuous variables were compared by means of the Spearman rank correlation coefficient. The Mann-Whitney U-test was used to compare variables grouped in relation to categories.

Results

The telomerase activity in normal skin (5 μ g protein extract) ranged from 5 to 50 CE, with a median of 20 CE (equivalent to 8 ng protein extract from L428 cells). This activity probably corresponds to the basic activity of parabasal proliferating cells in the epidermis and has to be considered in the interpretation of all other results because of the invariable presence of an epidermal component in the samples. The results are detailed in Table 2.

Table 2. Telomerase Activity (in 5 μ g Protein Extract), Ki-S11 and Ki-S2 Index in Common and Dysplastic Nevi and Malignant Melanomas

Diagnosis	Telomerase activity (CE) median [range]	Ki-S11 (%) median [range]	Ki-S2 median [range]
Common nevi	57.5 [10–350]	0.55 [0–2.1]	0.2 [0–0.9]
Dysplastic nevi	70 [15–750]	0.95 [0–2.6]	0.4 [0–1.5]
Malignant melanoma	885 [300–>5000]	9.75 [4.5–52.0]	2.85 [1.3–27.3]

In the entire series including nevi and melanomas, telomerase activity ranged from 10 to >5000 CE. It was significantly associated with the overall growth fraction as measured by the Ki-67 index ($r = 0.74$, $P < 0.0001$), and correlated even more closely with the percentage of Ki-S2 positive cells ($r = 0.80$, $P < 0.0001$). This relationship remained verified when nevi and melanomas were examined as separate groups. In melanomas, the correlation of telomerase activity was $r = 0.89$ with Ki-67, and $r = 0.93$ with Ki-S2 (both $P < 0.0001$). The association was not quite as close in nevi, with $r = 0.54$ for telomerase/Ki-67 and $r = 0.63$ for telomerase/Ki-S2, but nevertheless highly significant (both $P < 0.0001$). This may be due to the inevitable inclusion of the epidermal telomerase activity in the measurements, whereas the reactivity of keratinocytes with proliferation markers was not taken into account.

Nevi and melanomas exhibited clear-cut differences in terms of Ki-67 expression, Ki-S2 reactivity, and telomerase activity (all $P < 0.0001$). The lowest telomerase levels and proliferation indices were found in simple lentigos (Figure 2A). In dysplastic nevi (DN), there was a trend toward a higher proliferative activity and increased telomerase activity compared with common nevi (CN), but the difference was not statistically significant. However, five of 35 DN had a telomerase activity exceeding 200 CE, which was observed in only two of the 37 CN. Furthermore, telomerase activity was greater than 500 CE in two of the DN (Figure 2B), whereas none of the CN attained comparable values. Both of these DN belonged to patients with familial dysplastic nevus syndrome. Such high levels of telomerase activity were invariably associated with increased proliferation indices and, remarkably, with an augmented Ki-S2-to-Ki-67 ratio despite an overall correlation of $r = 0.97$ between the two markers in the whole series. In contrast, the nevi with high telomerase activity did not display any histological or cytological peculiarities compared with nevi of the same histological type but with significantly lower telomerase activity. Interestingly, of the seven nevi with telomerase activity levels above 200 CE, only four were atypical (dysplastic) both clinically and histologically, two displayed gross atypia without microscopic signs of dysplasia, and one was inconspicuous in every regard. Conversely, 13 clinically atypical nevi diagnosed as dysplastic nevi by histology exhibited telomerase activity levels below 50 CE, which is well within the range of the basic activity in normal skin.

In melanomas, there was a tendency toward increased telomerase activity in higher Clark's levels or tumors with deeper invasion. The same trend was observed with both proliferation markers, but none of the correlations achieved statistical significance. This may be attributable

to the small sample size examined. Nevertheless, the one melanoma *in situ* in our series contained a lower telomerase activity (600 CE) than any of the invasive melanomas, and the highest telomerase activity levels (>5000 CE) were found in the two cases of metastatic melanoma (Figure 2C). On the other hand, the correlation was obviously skewed by a case of level IV acrolentiginous melanoma with a low proliferative activity and another case of level IV melanoma of superficial spreading type with advanced regression, both of which contained only a relatively low telomerase activity (1300 and 1000 CE, respectively).

Figure 2. Graphic representation of telomerase activity as determined by capillary electrophoresis (y axis, relative activity; x axis, number of bases). **A:** Papillomatous dermal nevus with low telomerase activity (20 CE). **B:** Dysplastic nevus shown in Figure 1 (520 CE). **C:** Metastatic malignant melanoma with a telomerase activity greater than 5000 CE. Telomerase peaks are represented in relation to ITAS (black peak); because the latter tends to become undetectable in the presence of extreme telomerase levels, **C** corresponds to a 1:5 dilution of the sample (1 μ g protein), yielding a telomerase activity of 1250 CE.

Complete information on the clinical course could be obtained in all but one case. The median follow-up time was 57 months (46–72 months) for the nevi and 34 months (29–78 months) for the melanomas. None of the nevi recurred, and no occurrence of malignant melanoma was seen in these patients during the observation period.

The two patients with metastatic melanoma rapidly succumbed to widespread disease. Disease progression was seen in three of the remaining melanoma cases. One patient with Clark level IV melanoma of 4 mm thickness, Ki-67 and Ki-S2 indices of 23.5 and 9.7%, respectively, and a telomerase activity of 4500 CE developed lymph node metastasis and distant metastasis 2 years after surgical removal of the primary tumor and died 5 months later of the disease. Another patient with a level III melanoma of 0.89 mm thickness, respective Ki-67 and Ki-S2 indices of 13.5 and 5.7%, and a telomerase activity of 3000 CE developed lymph node metastasis 20 months after tumor excision and distant metastasis 12 months thereafter and survived for a further nine months before tumor-related death. A third patient with a level IV melanoma of 2 mm thickness and a high Ki-67 index of 23.5% but a Ki-S2 index of only 4.2% and a comparatively low telomerase activity of 2000 CE stayed disease-free for 51 months after primary surgery. Lymph node metastasis was shortly followed by distant metastasis, and both were surgically removed. A recurrent lymph node metastasis was recently excised, and the patient is alive without detectable residual disease 82 months after primary diagnosis. Interestingly, the proliferation indices and the telomerase activity in the last excised lymph node metastasis were almost identical to those of the primary tumor.

No progression was observed in the remaining melanoma cases, nearly all of which had lower levels of telomerase activity. Notably, one level IV melanoma of 4.1 mm thickness with a telomerase activity of 1300 CE did not progress to metastatic disease during an observation period of 38 months, despite the advanced T stage at diagnosis. One patient with a melanoma containing a telomerase activity of 2400 CE was lost to follow-up.

Discussion

The biology and natural history of melanocytic tumors are still incompletely understood. Although it is now widely recognized that patients with both high numbers of nevi and multiple moles with clinically atypical features are at increased risk for developing malignant melanoma,^{48–51} the biological significance of sporadic dysplastic nevi is a subject of persistent debates. The definitions of melanocytic dysplasia are mercurial and imprecise,⁵² and there are apparently no reliable histomorphological criteria that might allow one to confidently identify a melanocytic lesion as a precursor or risk marker for malignant melanoma.^{52,53} More pertinent information might, therefore, be gained by the assessment of biological parameters.

Immortality is a hallmark of malignant cells, and because the activation of telomerase is a prerequisite for cellular immortalization,^{54,55} we assessed the relative te-

lomerase activity in a variety of melanocytic lesions and compared it with the proliferative activity of the tumor cells. Our telomerase assay is robust with respect to false positive results,⁴⁴ and a deproteinization step, the integration of an internal amplification standard, and comparison with values of a calibration curve warrant a sufficient sensitivity and allow a reasonable quantification.^{44,45}

As expected, we found that the degree of telomerase activity discriminated neatly between benign and malignant lesions and correlated to some extent with tumor stage in malignant melanoma. The highest levels were found in the two metastatic melanomas, which represent an advanced stage in the evolution of the disease. In line with these observations, early metastasis tended to occur in the cases with high telomerase activity, whereas low telomerase levels were associated with absent or slow disease progression during our observation period. Furthermore, a melanoma *in situ*, which cannot be regarded as a full-blown malignancy, and a melanoma with advanced regressive change exhibited relatively low telomerase activity. In nevi, the activity was even markedly lower and did not overlap with the range seen in melanomas. This indicates that telomerase activity is likely to correlate closely with the biological properties of melanocytic proliferations.

In contrast, no statistically significant difference was found between histologically common and dysplastic nevi. Indeed, 13 of 35 DN contained virtually no telomerase activity, considering that the basic level in normal skin may attain almost 50 CE. On the other hand, three lesions that did not fulfill the histological criteria for dysplastic nevus exhibited comparatively high levels of telomerase activity. These findings suggest that the hitherto proposed diagnostic criteria insufficiently reflect the tumor biology in melanocytic lesions, and that careful correlations of biomarkers with morphological features in large case series might help to establish new standards.

Another aspect deserving consideration was the comparison of telomerase activity with cellular proliferation. In line with previous investigations,^{56,57} the proliferation indices were significantly different in benign and malignant lesions but failed to discriminate between common and dysplastic nevi. We observed a strong overall correlation between telomerase activity and both proliferation markers. Linear regression analysis even revealed a closer relationship with Ki-S2 than with Ki-S11, which is in keeping with the observation that, during cell cycle progression, telomerase activity peaks in the S phase^{58,59} compared with those of the other periods of the cell cycle.

Because of similar observations, it has been suggested that telomerase activity is a mere marker of cellular proliferation⁶⁰ that is not necessarily denotative of malignancy. However, although telomerase is unlikely to be activated in noncycling cells, not all proliferating cells express telomerase activity,^{10,61} which may first become apparent after immortalization.⁶² In this way, the activation of telomerase, notably in the absence of proportionally increased proliferative activity, might reflect a step toward immortalization, although transformation to full-

blown malignancy may require additional molecular events.⁶³ Considering that the correlation between telomerase activity and proliferation was noticeably weaker in nevi than in melanomas, the activation of telomerase might be an indicator of early malignant transformation in melanocytic tumors.

Consistent with this idea, our clinicopathological correlations imply that the biological information gained from telomerase assays extends beyond that provided by gross and microscopic features as well as proliferation measurements. Indeed, a case of thin level III melanoma with an intermediate Ki-67 index but high telomerase activity followed a fatal course, whereas no progression was observed in a case of thick melanoma with a higher Ki-67 index but low telomerase activity. This is well in line with our recent observations in a series of endometrioid adenocarcinomas of the uterus (Bonatz et al, manuscript submitted, for publication), showing that telomerase activity correlates poorly with the Ki-67 index while being closely associated with the clinical outcome. Moreover, the degree of telomerase activation was connected with the rate of cells reentering S phase rather than with the overall growth fraction, indicating an impaired cell cycle control. Although further investigations are needed to establish the relationship between telomerase and malignancy, it appears that telomerase activation is not solely an intrinsic property of proliferating cells.

It emerges from our data that proliferation measurements do not necessarily allow conclusions about the degree of telomerase activity. repp86 expression, although measuring a different quality, appears to correlate more tightly with telomerase activity than the overall growth fraction. However, considering the minimal variations of the Ki-S2 index in nevi and the much wider range of telomerase activity levels, the latter present at least a higher resolution power and might be a more sensitive marker. Therefore, the combined analysis of proliferation characteristics and telomerase activity might provide deeper insights into tumor biology.

To our knowledge, only one comparative analysis of telomerase activity in melanocytic lesions has been published so far.⁶⁴ On the basis of apparently significant differences in relative telomerase activity, these authors concluded that dysplastic nevi represent an intermediate stage in a putative progression from benign nevi to malignant melanomas. We believe that, as interesting as their data are, they should be interpreted with caution. Besides the lack of validation by integration of an amplification standard, the reported activity levels in nevi are within a range where, in our experience, the PCR enzyme-linked immunosorbent assay kit used in this study does not grant a safe resolution. Nevertheless, although we were not able to ascertain statistical significance, our results show a similar trend. Notably, the two nevi with the highest relative telomerase activity had the histological features of dysplastic nevi and belonged to patients with familial dysplastic nevus syndrome. This syndrome, also known as familial atypical multiple mole syndrome (FAMMS), is now commonly considered to be a condition predisposing to malignant melanoma.^{65–68} Furthermore, it appears that melanomas can originate from those nevi

with gross and microscopic atypia.^{69,70} High levels of telomerase activity might therefore reflect an early step in the process of malignant transformation undetectable by morphological examination.

One major shortcoming of this study is that the evidence produced is largely circumstantial. Nevi, *in situ* melanocytic proliferations, and even the majority of thin melanomas are cured by simple excision, and what might occur if the lesions were allowed to remain is conjectural. Another drawback is that telomerase activity cannot yet be correlated to specific cell populations. Although providing no information on the biological activity of telomerase, *in situ* hybridization for hTERT mRNA, encoding the catalytic subunit of the telomerase complex,²¹ might supply valuable information in this regard.

Malignant melanomas are not infrequently found in association with preexisting nevi,⁷¹ but definite proofs of a direct transition are lacking. However, biological criteria seem to indicate that malignant transformation may occur in benign nevi^{72,73} and that early steps in this pathway may not be discernible by conventional morphology, which is supported by our findings. Further investigations using both activity assays and *in situ* PCR techniques for telomerase detection are needed to establish the relevance of biomarkers that in future might usefully complement morphological examination. So far, our results imply that biological alterations may occur in nevi irrespective of morphological changes and that both proliferation measurements and telomerase assays may be helpful in discriminating between benign and malignant lesions. Moreover, the correlation with clinical data points to a possible prognostic significance of the relative telomerase activity.

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