Inhibition of tumor angiogenesis by cannabinoids[1]

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SPECIFIC AIMS

Cannabinoids, the active components of marijuana and their derivatives, inhibit tumor growth in animal models, but the mechanism of their anti-tumoral action in vivo is still unclear. Because the generation of a new vascular supply (angiogenesis) is causally involved in the progression of the majority of solid tumors, the aim of this study was to test whether cannabinoids inhibit tumor angiogenesis.

PRINCIPAL FINDINGS

1. Cannabinoid administration inhibits tumor angiogenesis

We induced malignant tumors in immune-deficient mice by subcutaneous flank inoculation of rat C6 glioma cells or human grade IV astrocytoma cells and injected them for 8 days with vehicle or the nonpsychoactive CB_2 cannabinoid agonist JWH-133 at 50 μg/day. We found that tumors from cannabinoid-treated animals were smaller and paler than controls (**Fig. 1*A, B***). Analysis of the vasculature by immunostaining of CD31, an endothelial cell marker, revealed no significant effect of JWH-133 on microvascular count (number of blood vessels per unit area) in the tumors (data not shown). However, cannabinoid administration turned the microvascular hyperplasia of control tumors (Fig. 1*C, E*) to a pattern of blood vessels characterized predominantly by very small and narrow capillaries (Fig. 1*D, F*). Moreover, whereas control tumors showed a fractionated immunostaining of smooth muscle α-actin (SMA) (Fig. 1*G*), a smooth muscle cell and pericyte marker, in cannabinoid-treated tumors SMA-positive cells had a mature appearance and remained closely investing the endothelial wall (Fig. 1*H*). We next examined whether these changes in tumor vascularization led to actual differences in vascular functionality as assessed by a vascular permeability assay. Tumor-bearing animals were anesthetized and Evans blue (1% in PBS, 100 μL/mouse) was injected into the tail vein. We found that dye accessibility to the tumors was much lower in cannabinoid-treated animals than in controls. Thus, analysis of the in situ tumor pictures showed that the dye extravasation area in the tumor relative to total tumor area was 84 ± 11% in control animals and 15 ± 7% in JWH-133-treated animals for C6 cell gliomas (n=4, P<0.01) and 88 ± 19% in control animals and 21 ± 11% in JWH-133-treated animals for human astrocytomas (n=4, P<0.01). All these observations indicate that the vascular network of actively growing control tumors is large, plastic, and leaky whereas that of slowly growing cannabinoid-treated tumors is small, differentiated, and impermeable.

2. Cannabinoid administration inhibits vascular endothelial cell migration and survival

We examined the direct effect of cannabinoids on vascular endothelial cell migration and survival. Western blot analyses showed that primary human umbilical vein endothelial cells (HUVEC) express the two subtypes of cannabinoid receptors (data not shown). In cell culture experiments, JWH-133 (25 nM) inhibited HUVEC migration as assessed by the Boyden chamber method (57±19% of control, n=6, P<0.01) and induced HUVEC apoptosis as determined by oligonucleosomal DNA fragmentation and TUNEL staining (data not shown).

3. Cannabinoid administration inhibits tumor expression of proangiogenic factors and improves other markers of tumor malignancy

Tumors promote angiogenesis by secreting proangiogenic cytokines, which induce the formation of blood

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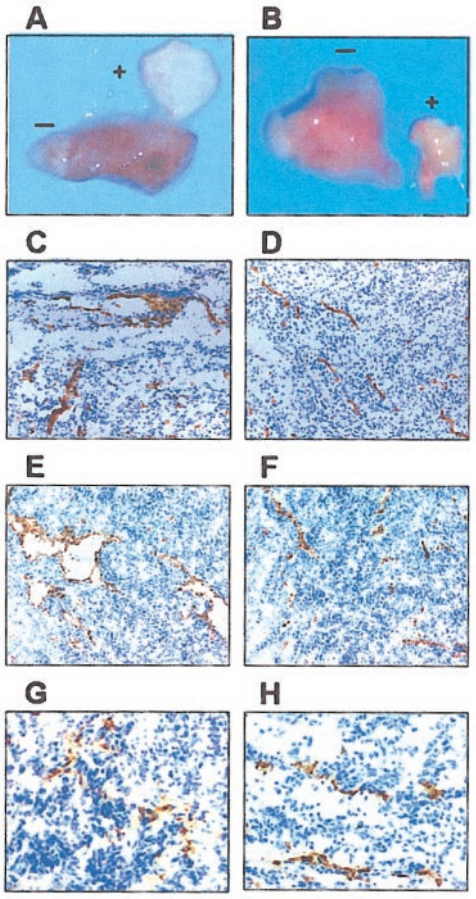

Figure 1. Cannabinoid administration inhibits tumor angiogenesis. *A, B)* General appearance of dissected tumors. Animals bearing C6 cell gliomas (*A*) or human astrocytomas (*B*) were treated without (−) or with (+) JWH-133. Note the small size and pale color of cannabinoid-treated tumors. *C–F)* CD31 staining showing the blood vessel pattern in the tumors. Animals bearing C6 cell gliomas (*C, D*) or human astrocytomas (*E, F*) were treated without (*C, E*) or with (*D, F*) JWH-133. Note the small size of the vessels in cannabinoid-treated tumors. *G, H*) SMA staining showing the pattern of smooth muscle cells and pericytes in the tumor blood vessels. Animals bearing human astrocytomas were treated without (*G*) or with (*H*) JWH-133. Note the high perivascular coverage in cannabinoid-treated tumors. Representative tumors are shown in each panel. Similar data were obtained in 7–9 (*A, B*) or 3–4 (*C–H*) additional animals.

vessels. We next tested whether JWH-133 inhibits angiogenesis not only by targeting vascular endothelial cells directly, but also by interfering with proangiogenic factor expression. Cannabinoid administration markedly reduced the expression of vascular endothelial growth factor (VEGF), the most potent and ubiquitous proangiogenic factor, and of angiopoietin 2 (Ang2), which also contributes to the angiogenic process by preventing vessel maturation (**Fig. 2*A***). In contrast, Ang1 expression was not affected by JWH-133, in line with previous reports showing that this cytokine is not greatly involved in the pathogenesis of gliomas. Cannabinoid treatment decreased as well the activity and expression of matrix metalloproteinase-2 (MMP-2), a proteolytic enzyme that allows tissue breakdown and remodeling during invasive angiogenesis, whereas tissue inhibitor of metalloproteinase-2 (TIMP-2) expression remained unaffected (Fig. 2*B*). Moreover, areas of necrosis with palisading nuclei, a characteristic of highly malignant tumors, appeared in control but not in cannabinoid-treated tumors (Fig. 2*C*).

CONCLUSIONS AND SIGNIFICANCE

Cannabinoids inhibit tumor growth in animal models, but the mechanism of their anti-tumoral action in vivo is still unclear. Here we report that cannabinoids inhibit tumor angiogenesis in vivo and that at least two mechanisms may be involved in this cannabinoid action: direct inhibition of vascular endothelial cell migration and survival, and suppression of proangiogenic factor and MMP expression in the tumors. A parsimonious interpretation of these and our previous findings is that cannabinoids inhibit tumor growth by activating cannabinoid receptors in both vascular endothelial cells and tumor cells. By inhibiting vascular endothelial cell migration and survival, cannabinoids would directly prevent blood vessel formation. By targeting tumor cells, cannabinoids would induce their apoptosis and also suppress proangiogenic factor and MMP production, further blocking tumor growth and angiogenesis (**Fig. 3**).

VEGF and Ang2 are essential for the vascularization of many types of tumors. Coinciding with high expres-

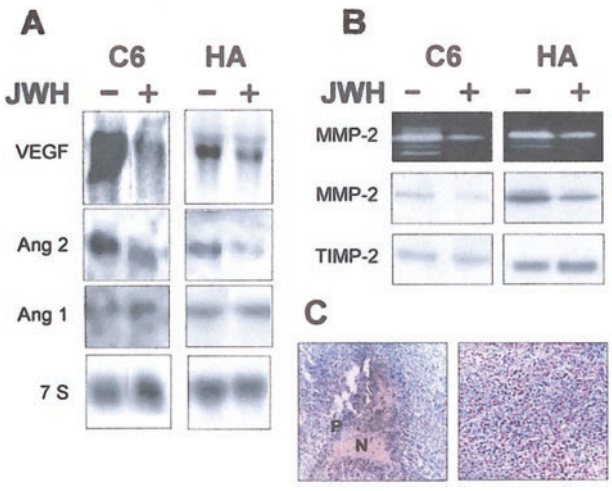

Figure 2. Cannabinoid administration inhibits expression of proangiogenic factors and improves other markers of tumor malignancy. *A, B)* Animals bearing C6 cell gliomas (C6) or human astrocytomas (HA) were treated without (−) or with (+) JWH-133. *A)* Expression of VEGF, Ang1 and Ang2 in the tumors as determined by Northern blot. 7S RNA was used as loading control. *B)* MMP-2 activity (zymogram, top), MMP-2 expression (Western blot, middle) and TIMP-2 expression (Western blot, bottom) in the tumors. *C)* Necrosis (N) with palisading nuclei (P) in C6 cell gliomas from control (left) but not JWH-133-treated (right) animals. Representative tumors are shown in each panel. Similar data were obtained in 2–4 additional animals.

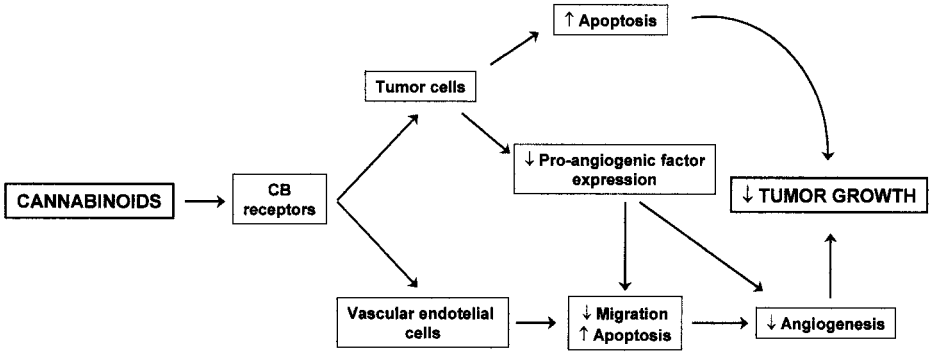

Figure 3. Schematic diagram depicting possible mechanisms involved in cannabinoid anti-tumoral action.

sion of these major proangiogenic factors, vascular profiles of actively growing tumors are usually characterized by enhanced leakage, weak association with perivascular cells, and abnormal deposition of extracellular matrix. This is precisely what we observe in tumors from control animals (i.e., high VEGF and Ang2 expression associated with increased vascular permeability, fractionated SMA staining, and enhanced MMP-2 activity), just the opposite of what occurs in cannabinoid-treated tumors.

Two different cannabinoid receptors have been characterized and cloned from mammalian tissues: the "central" CB_1 receptor, which is responsible for cannabinoid psychoactivity, and the "peripheral" CB_2 receptor, which is unrelated to cannabinoid psychoactivity. We show here that vascular endothelial cells, like glioma cells, express functional CB_2 receptors. Hence the present report, together with the possible implication of CB_2 or CB_2-like receptors in the control of, for example, peripheral pain and multiple sclerosis-linked spasticity, opens the attractive possibility of finding cannabinoid-based therapeutic strategies devoid of undesirable CB_1-mediated psychotropic side effects.

Because active angiogenesis is causally involved in the progression of the majority of solid tumors, considerable effort is being made in developing effective anti-angiogenic drugs to treat cancer. In the context of the renaissance in the study of the therapeutic effects of cannabinoids, our findings show that these compounds may be considered promising anti-tumoral agents as they inhibit tumor angiogenesis and growth in vivo with no significant side effects. This report provides a mechanistic basis for the anti-tumoral action of cannabinoids and a novel pharmacological target for cannabinoid-based anti-tumoral therapies. Nonetheless, further research is required to elucidate the molecular mechanisms by which cannabinoids control proangiogenic factor and MMP production and how these molecules in turn affect blood vessel formation. FJ